Biochimica et Biophysica Acta 1832 (2013) 1807–1826

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Review

Human pathology in NCL ☆

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ARTICLE INFO

Article history:
Received 3 July 2012
Received in revised form 9 November 2012
Accepted 20 November 2012
Available online 29 November 2012

Keywords:
Electron microscopy
Brain
Extracerebral tissues
Granular osmiophilic deposits
Curvilinear
Fingerprint

ABSTRACT

In childhood the neuronal ceroid lipofuscinoses (NCL) are the most frequent lysosomal diseases and the most frequent neurodegenerative diseases but, in adulthood, they represent a small fraction among the neurodegenerative diseases. Their morphology is marked by: (i) loss of neurons, foremost in the cerebral and cerebellar cortices resulting in cerebral and cerebellar atrophy; (ii) an almost ubiquitous accumulation of lipopigments in nerve cells, but also in extracerebral tissues. Loss of cortical neurons is selective, indiscriminate depletion in early childhood forms occurring only at an advanced stage, whereas loss of neurons in subcortical grey-matter regions has not been quantitatively documented. Among the fourteen different forms of NCL described to date, CLN1 and CLN10 are marked by granular lipopigments, CLN2 by curvilinear profiles (CVPs), CLN3 by fingerprint profiles (FPPs), and other forms by a combination of these features. Among extracerebral tissues, lymphocytes, skin, rectum, skeletal muscle and, occasionally, conjunctiva are possible guiding targets for diagnostic identification, the precise type of NCL then requiring molecular analysis within the clinical and morphological context. Autosomal-recessive adult NCL has been linked molecularly to different childhood forms, i.e. CLN1, CLN5, and CLN6, whilst autosomal-dominant adult NCL, now designated as CLN4, is caused by a newly identified separate gene, *DNAJC5*. This article is part of a Special Issue entitled: The Neuronal Ceroid Lipofuscinoses or Batten Disease.

1. Introduction

Morphologically, the neuronal ceroid lipofuscinoses (NCL) are marked by two features: (i) loss of nerve cells in the brain and retina and (ii) accumulation of intracellular lipopigments, especially in neurons. Depletion of neurons in NCL affects primarily the cerebral and cerebellar cortices; quantifying studies concerning subcortical grey matter regions and nuclei have not yet been performed but, if present at all, depletion is more subtle. The degree of neuronal depletion is inversely proportional to the length of the disease and proportional to the onset of clinical symptoms. Loss of nerve cells outside of the brain and the retina is, likewise, not well-documented. The reason for the preferential loss of cortical neurons in NCL is still ill-understood. Moreover, the examiner of NCL patients' central nervous system (CNS) has to be aware of the fact that at autopsy the pathology in the CNS and the retina is at end-stage, allowing little analysis of the morphological evolution of the condition such as early and/or preferential loss of neuronal populations and types of neurons in the CNS. Even so, in the brains of juvenile and adult NCL patients it appears that small neurons in the cortex are earlier and more severely depleted than the larger, e.g. pyramidal, ones whilst accumulation of lipopigments is more prominent in large neurons, again pyramidal cells of the cerebral cortex — but not in large Purkinje cells of the cerebellum. Post-mortem studies have revealed that in the cerebral cortex neuronal degeneration starts at the dendritic profile inducing a microglial reaction whilst the neuronal perikaryon is still present. In the retina, neuronal depletion commences in the outer layer, i.e. in the photoreceptor outer segments, followed by that of the inner segments and then, of nerve cell bodies from where the process proceeds inward towards the ganglionic layer. Pigmented epithelial

Abbreviations: ADANCL, autosomal-dominant adult NCL; ANCL, adult-onset NCL; CNS, central nervous system; *CTSD*, gene encoding cathepsin D; CVP(s), curvilinear profile(s); CVS, chorion villus sample; EM, transmission electron microscopy; EP, evoked potentials; EPMR, progressive epilepsy with mental retardation; ER, endoplasmic reticulum; ERG, electroretinogram; FPP(s), fingerprint profile(s); GROD(s), granular osmiophilic deposit(s); JNCL, juvenile NCL; LFB, luxol fast blue; LINCL, late-infantile NCL; LM, light microscopy; MPS IIIA, mucopolysaccharidosis IIIA; NCL, neuronal ceroid lipofuscinosis; PAS, periodic acid-Schiff; PME, progressive myoclonus epilepsy; PPT1, palmitoyl protein thioesterase 1; RLP(s), rectilinear profile(s); SAP(s), sphingolipid activator protein(s); SCMAS, subunit C of mitochondrial ATP synthase; TPP1, tripeptidyl peptidase 1; vLINCL, variant late-infantile NCL

☆ This article is part of a Special Issue entitled: The Neuronal Ceroid Lipofuscinoses or Batten Disease.

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http://dx.doi.org/10.1016/j.bbadis.2012.11.014

cells, filled with lipopigments, may proliferate and enter the depleted neuronal retinal layers.

Whilst a complete picture of the CNS pathology can only be ascertained at autopsy, many diagnostic studies by brain biopsy, usually of the cerebral cortex, have focused on the accumulation of lipopigments as a diagnostic marker but they have contributed little to our understanding of true structural damage. Most diagnostic brain biopsy investigations were carried out before wide-spread accumulation of lipopigments in extracerebral tissues had been recognised, and have now been superseded by those of skin, rectum, skeletal muscle and even blood lymphocytes. This shift in diagnostic material from CNS to non-CNS tissues had already occurred in the pre-molecular era and, thus, before the advent of immunohistochemistry which might otherwise have gainfully been applied to biopsied brain tissue. Thus, retrospective investigations on archival CNS tissue may provide new information, especially on early-onset NCL but, to our knowledge, no such study has been performed. A large ultrastructural spectrum of lipopigment morphology was identified in brain biopsies but this has not been significantly further expanded by studies on extracerebral tissues. These studies were executed largely by transmission electron microscopy (EM), requiring rapid fixation in glutaraldehyde for optimum morphological preservation, rendering further investigative procedures almost impossible. Hence, there is a potential gap in our understanding of detail that might otherwise be obtainable by immunohistochemistry such as the identity of new proteins accruing within lipopigments. Likewise, ultrastructural study of lipopigments in the brain, apart from those in cerebro-cortical diagnostic biopsies, has only scantily been investigated [1]. Lipopigments as lysosomal residual bodies, with the intralysosomal metabolism almost completed, are rather resistant to agonal and post mortem autolysis, thus comprehensive ultrastructural studies of CNS lipopigments in subcortical grey and white matter as well as the retina may prove useful.

These considerations attest to the view that, despite a decline in morphological studies on the CNS in NCL, there are still areas that might be gainfully explored with modern immunohistochemical techniques in the human NCL brain.

2. Neuropathology of the central nervous system

2.1. Childhood forms of NCL

Whilst many independent studies published over several decades have reported on CNS neuropathology in the classical childhood forms of NCL, i.e. CLN1, CLN2, and CLN3, studies concerning CNS neuropathology in the more recently delineated NCL forms are few and not always comprehensive. For instance, of the two forms of CLN8, the late-infantile variant and the northern epilepsy (EPMR), only few of the latter group have been reported [2]. Likewise, of CLN10, constituting a congenital form as well as a post-infantile one, only publications of the congenital form are available for CNS tissue from the pre-molecular era [3].

2.1.1. Macroscopy

Macroscopically, the brain is almost always reduced in size, frequently resulting in microcephaly in CLN1 and CLN10, although this is not seen in all patients in the EPMR variant of CLN8 who reached adulthood [2]. Increasing severity of brain atrophy is apparent not only between early and late onset forms of childhood NCL, i.e. CLN1 and CLN3, but also with progression of the disease in the same CLN group, e.g. CLN6, having for instance a brain weight of 883 g (control: 1263 g) at age 7 years and of 450 g (control: 1400 g) at age 13 years [4]. Reduction in brain weight is chiefly due to cerebral cortical atrophy accompanied by a reduction in white matter, and enlargement of cerebrospinal fluid-carrying spaces, the subarachnoid space and the ventricles, and to a varying degree, atrophy of the cerebellum, particularly in CLN1 and CLN2 but also, variably, in the other childhood forms.

2.1.2. Light microscopy

Preferential loss of neurons has been described for small neurons of layer II in CLN3 [5,6], in layers III and V in CLN2 [7], in mid-layers in CLN6 [4], and in layer V in CLN7 [8]. Detailed microscopic and immunohistochemical studies performed on the human hippocampus in childhood forms of NCL [9] show relatively minor neuronal depletion and lipopigment formation in the CA-1 sector as well as sparing of calretinin-positive interneurons and microglial activation. In juvenile NCL (JNCL), the substantia nigra often lacks pigment because of neuronal loss which may explain Parkinsonian features encountered in these patients. There is loss of Purkinje cells and granular cells in the cerebellum, of granular cells in the dentate nucleus, whilst subcortical grey matter in the cerebrum, the brainstem and spinal cord is not strikingly depleted of nerve cell bodies. Depletion of neurons is accompanied and followed by activation of microglia, the presence of lipopigment-containing macrophages and astrocytes, the latter forming a glial fibrillary network or fibrillar gliosis. Activated microglia in areas where neuronal loss is not yet obvious may indicate the commencement of morphological damage indicating degeneration of dendritic processes at the periphery of neurons.

Atrophy of the retina is particularly pronounced in CLN1 and CLN3, less so in CLN2, but also present in the late-infantile variants CLN5 [7], CLN6 [4] and CLN7 [8] whilst apparently nothing has been described of the retinal pathology in CLN8, CLN9 [10], and CLN10. Occasionally, the progression of atrophy from the photoreceptor layer to the ganglionic layer is not apparent, such as in patients with CLN5 [7]. Gliosis of varying degrees and the presence of macrophages mark the atrophic retina.

Conspicuous accumulation of lipopigments occurs in nerve cell bodies, often extending into the proximal axonal segments or axon hillocks, terminal axonal spindles or meganeurites. By light microscopy (LM), the lipopigments appear brownish in haematoxylin–eosin-stained sections, imparting a mild brownish hue to the grey matter at gross inspection. These lipopigments stain with periodic acid-Schiff (PAS) and appear bluish when stained with luxol-fast blue (LFB). Their lipid content may also give a black appearance in Sudan black stain and a reddish appearance in oil red-O stain. However, the 'lipid droplets' may not be or may partially not be lipids as revealed by lipofuscin studies in equine thyroid glands: "We looked at lipid droplets in lipofuscin in the equine thyroid gland. Here similar structures are not lipid but caused by infusion of colloid droplets (i.e. soluble protein) with the hydrophobic condensed matrix that made up the lipofuscin pigment. As with the thyroid pigment it may just be a watery vesicle partly embedded in the proteolipid/lipid matrix with the oily appearance due to the varying density of the matrix as seen through the thickness of the section." (Robert D. Jolly, personal electronic communication, October 18, 2012, to co-author H.H.G.).

2.1.3. Electron microscopy

Autofluorescence and EM may also reveal accumulation of lipopigments in dendritic processes. Cerebrocortical, subcortical, and cerebellar neurons as well as those of the brainstem and the spinal cord are varyingly affected, with granular ultrastructure in CLN1 and CLN10, curvilinear profiles (CVPs) in CLN2 and mixed CVP, rectilinear (RLP), and FPPs in the remaining CLN forms. Astrocytes, ependymal cells, and choroid plexus epithelial cells may harbour lipopigments, oligodendroglia are said to be sparsely affected but phagocytosed lipopigments are evident in macrophages. Neuronal perikarya of dorsal root ganglia and other peripheral nerve cell clusters may also display lamellar inclusions resembling the membranous cytoplasmic bodies seen in the gangliosidoses [1].

2.1.4. Immunohistochemistry

Immunohistochemistry reveals the presence of abundant sphingolipid activator proteins (SAPs) in CLN1 and CLN10 whilst subunit C of mitochondrial ATP synthase (SCMAS) prevails in the lipopigments of other CLN forms. A minor component of either SAPs or SCMAS may also be encountered in the forms of CLN where the other component dominates the picture. The amyloid precursor protein and beta-A4 amyloid protein may also, though rarely, be seen within lipopigments [2,11]. Lysosomal proteins, such as palmitoyl protein thioesterase 1 (PPT1), tripeptidyl peptidase 1 (TPP1), and cathepsin D (pCTSD) may be encountered along with the lipopigments, except in the CLN forms where they are deficient by mutations, i.e. PPT1 in CLN1, TPP1 in CLN2, and CTSD in CLN10.

3. Adult NCL

Several aspects render adult NCL a special form among the different age-related forms of NCL. It is much rarer than the childhood forms. On a hereditary basis, it may follow autosomal-recessive or autosomal-dominant traits of inheritance whilst sporadic cases have also been encountered. A precise nosological diagnosis in non-recessive forms requires autopsy. Whilst all childhood forms of NCL follow an autosomal-recessive mode of inheritance, families with autosomal-dominant adult NCL have now been described, some of them associated with mutations in a newly identified gene, *DNAJC5*. One of the neuropathological hallmarks of childhood NCL, loss of neurons, may not be quite so obvious in adult NCL. Final nosological diagnosis of adult NCL may remain equivocal in certain patients;CNS neurons which have accumulated considerable amounts of lipopigments, may not easily be identified as having adult NCL since, with increasing age of the patient, lipopigments may accrue in non-NCL lysosomal diseases, such as mucopolysaccharidoses or GM_1-gangliosidosis. This uncertainty or even inaccuracy may increase when only biopsy data from extracerebral tissue are available for diagnosis.

Recently, adult patients with familial NCL have been found to have homozygous or compound heterozygous mutations and have, therefore, been genetically assigned to childhood forms of NCL. When such patients with pre-adult onset of equivocal symptoms and protracted forms have to be distinguished from those with true adult-NCL, the absence of visual deficits (clinically and fundoscopically the hallmark of true adult-onset NCL (ANCL)) may aid in further identifying adult NCL since even subtle retinal abnormalities have not been documented in adult NCL of the CLN1 type [12].

3.1. Adult autosomal-recessive type of NCL

Whilst adult-NCL families, usually siblings, suggestive of having autosomal-recessive inheritance have been described in the premolecular era, those successfully identified with homozygous or compound heterozygous mutations belong to CLN1 (although patients with mutations in the *CLN1* gene and lack of PPT1 enzyme activities have been diagnosed without autopsy), CLN5 [13], and CLN6 [14,15]. Although clinical abnormalities and evidence of retinal atrophy are absent, retinal neuronal perikarya may accumulate NCL-specific lipopigments [16,17], even in CLN5 adult NCL [13].

The second neuropathological hallmark of childhood NCL, the intraneuronal accretion of lipopigments, is also the hallmark of adult NCL, enlarging the neuronal perikarya and often forming proximal axon spindles or meganeurites replete with lipopigments. Even in the cerebral and cerebellar cortices neuronal loss may be mild or minimal and, thus, major gross atrophy of the brain may be absent. FPPs may be conspicuous within the neuronal lipopigments, and have been reported both at post mortem [13] and in brain biopsy [18]. A granular component is also often present whilst CVPs are rare or absent. The lipopigments contain, by immunohistochemistry, SCMAS and, to a lower degree, SAPs whilst beta-A4 amyloid as well as amyloid precursor protein have also been demonstrated [10].

The *SGSH* and *CLCL6* genes found in mucopolysaccharidosis IIIA (MPS IIIA) and a mouse model for lysosomal storage [19] respectively, have been assigned to NCL mutations but this link remains controversial. A patient who was compound heterozygous, i.e. with E355K and S298P mutations, had too little morphological details reported [20] to clearly identify the ultrastructure of the lysosomal residual bodies, being either vacuolar as in MPS IIIA, or of lipopigment nature, as in NCL. The combination of MPS IIIA and accretion of lipopigments in the cerebral cortex at autopsy, strikingly like in NCL, has previously been reported [11]. Likewise, the concomitant accumulation of lipopigments has also been encountered in other lysosomal storage diseases. Of the reported ANCL patients with only single heterozygous *CLCL6* mutations [19], one was reported without any morphological details whilst the second patient was later found to have compound heterozygous mutations in the *CLN5* gene [13]. This suggests that at least the second case, with a sole heterozygous mutation in the *CLCL6* gene, might have just been a carrier of a heterozygous *CLCL6* gene but not an NCL patient.

Recently four siblings who had NCL and Parkinsonian features were found to have the homozygous mutation in the *ATP13A2* gene found in CLN12. Autopsy studies revealed NCL-specific lipopigment accumulation in neuronal and glial cells of the cerebral and cerebellar cortices, in the basal ganglia and in the retina [21].

3.2. Adult autosomal-dominant type of NCL

To date, only adult familial NCL has been reported to carry an autosomal-dominant trait of inheritance. The bases for diagnosing such patients with autosomal-dominant adult NCL (ADANCL) are clinical symptoms and accretion of lipopigments in neuronal perikarya, very rarely in extracerebral tissues and, then, often difficult to distinguish from regular lipofuscin. At autopsy, the brain weight may be reduced (although not always [22]), below 1200 g [23] or, rarely, even under 900 g [24], whilst the cerebellum may be severely atrophic. Loss of cerebrocortical neurons is not always visible. However, Purkinje and granular cells in the cerebellum may be severely reduced in number. Repeated studies have also shown that neurons of the substantia nigra are depleted [23,24], and there is an accompanying loss of neuromelanin. Sometimes, loss of neurons in the substantia nigra was considered severe [25], consistent with the morphology associated with clinical Parkinsonian features. Neuronal loss was also observed in cerebellar nuclei and the inferior olives [25].

Neuronal depletion appears to lead to cellular and fibrillar astrocytosis. Lipopigments, characterised by autofluorescence and PAS-positive staining, were encountered in innumerable neurons of the cerebral cortex, subcortical grey matter of the brain and brainstem as well as the spinal cord whilst cerebellar neurons were less heavily affected. Apart from neuronal perikarya, proximal axonal segments were also filled with lipopigments as were occasional Purkinje cell dendrites [24]. Lipopigment formation has been encountered in selective neuronal populations; the CA-e region of the hippocampus and in neurons of the fascia dentata. In some patients lipopigments formed larger so-called myoclonus bodies [22,26]. Neurofibrillary tangles and amyloid plaques were largely missing although, occasionally, can be demonstrated with tau-antibodies in neurons of the cerebellar nuclei and substantia nigra [24].

3.3. Immunohistochemistry

By immunohistochemistry, SAPs were detected regularly in the lipopigments and, more rarely, SCMAS [24]. Increased microglial activity was seen in several subcortical grey matter areas, such as basal ganglia, thalamus, inferior olives, and brainstem nuclei [24].

3.4. Electron microscopy

By EM, the overwhelming pattern was that of granular osmiophilic deposits (GRODs) but, occasionally, also FPPs [24,25,27].

When successfully tested by molecular analysis, mutations in the *DNAJC5* gene were found [22–29]. Retinopathological data in ADANCL have not yet been reported.

4. Pathology of extracerebral tissues

4.1. Historical annotations

Advances in histochemistry and EM of brain biopsies for diagnostic purposes in the late 1960s and early 1970s led to the identification of NCL as an independent disease group among the storage diseases of childhood [30,31]. This was followed by a classification of NCL based on the age of clinical onset and cytosome ultrastructure [32]. At the same time, EM investigations of extra-cerebral tissues in neurodegenerative diseases became feasible, and proved particularly useful for NCLs (as compared to other storage diseases (reviewed in [33])).

The first report on the involvement of extracerebral tissues in NCL was by Joosten et al. in 1973 [34], who examined sural nerve biopsies of 2 children affected with "amaurotic idiocy". In the same year, cytoplasmic inclusions were reported in the vermiform appendix and skeletal muscle of 10 NCL patients [35], and subsequently the usefulness of muscle biopsies in NCL was reviewed by Goebel et al. [36]. The search for straightforward, easy-to-perform and less invasive diagnostic approaches led to ultrastructural examination of circulating lymphocytes in late-infantile NCL (LINCL) [37], and to the study of vacuolated lymphocytes in JNCL [38]. In 1974, successful EM diagnosis was reported from analysis of skin biopsies [39,40], which became thereafter the most common diagnostic instrument for NCL and a number of other neurodegenerative diseases of childhood. Rectal biopsy provides ready access to neuronal tissue in the myenteric ganglia. The assessment of these by histological and histochemical methods proved valid, and this led to the introduction of EM analysis of rectal biopsies in NCL [41,42] which overcame difficulties in differentiating by LM between storage material and lipofuscin. This approach was very useful since it provided evidence of accumulated storage product within the cytoplasm of extracerebral neuronal cells, and it became recommended for selected cases in which EM analysis of other tissues was not helpful. Moreover it contributed to the abandonment of brain biopsies, at least in neurodegenerative childhood disorders. Then, the classification of NCL was expanded by the recognition of subsets of patients by age of onset and clinical course (infantile and early-juvenile onset) as well as by new cytosome patterns (GRODs and mixed cytosomes, respectively) [43,44]. The availability of molecular tools by the end of the 1990s and the progressive identification of new NCL genes predicted a decline in the ultrastructural approach to diagnosis in NCL. However, ultrastructural examination of extracerebral tissues is still necessary in atypical cases, particularly the late-infantile variants (vLINCL), and the relatively stable figures (10–15%) of molecularly undetermined cases. This is also particularly true for the ultrastructural investigation of rectal biopsies in ANCL because the detection of specific storage might be uncertain or poor, or even missing in other extracerebral tissues [45]. Therefore, the correlations among clinical features, genetic data, and EM findings still represent useful tools to improve our knowledge and understanding of the pathobiology of this disease group [46]. Accordingly, ultrastructural analysis of extracerebral tissues in the diagnosis of NCL has been taken into account in a recent diagnostic algorithm and included within an axial system of classification [47].

4.2. General considerations

In this review we mainly focus on the EM features of skin biopsies and blood lymphocytes, which are the best characterised extracerebral tissues for ultrastructural investigations in NCL; however, we have also taken into account the findings from other biopsied tissues, such as the rectum and, to a lesser extent, skeletal muscle. In extracerebral tissues, cytosomes show the same patho-morphological and ultrastructural characteristics as observed in CNS. Autofluorescent, PAS and Sudan black B-positive granules, resistant to lipid solvents, accumulate in the cytoplasm of most cells. Interestingly, SCMAS is the major protein storage detected in all NCL forms (other than CLN1) identified so far. It is associated with more variable EM features, CVPs, RLPs or FPPs, reminiscent of the accumulation of adjacent membranes, the spatial distribution of which is related to the physico-chemical properties of the environment in which the abnormal material is stored [48]. Conversely, SAPs are the main storage of CLN1 only, and are associated with the GROD pattern, detected in this form.

EM studies of lymphocytes (obtained from gradient separation of blood samples, subsequently fixed and embedded) are particularly recommended for the evaluation of patients clinically referred to as having probable CLN1, CLN2 or CLN3, whose prevalent cytosomes (GRODs, CVPs, FPPs, and vacuoles, respectively) can easily be detected and can predict the mutated gene. In other NCL forms, namely vLINCL, EM lymphocyte analysis may be less useful since lysosomal storage is relatively nonspecific or scant when compared with the mixed patterns better detected in skin cells (Fig. 1). Lastly, the homogeneous lymphocyte cell population examined allows a semi-quantitative analysis, which can be followed along the course of the disease, and whose variations may represent a potential biomarker of disease progression.

Several advantages are obtained by examining a skin biopsy. First of all, heterogeneous cell populations can be observed in skin tissue and some cell type specificities can be recognised according to the NCL form: for instance CVPs commonly crowd the smooth muscle cells in CLN2, whereas vascular smooth muscle cells seem to be the site of choice to detect the scarcely represented FPP in ANCL [49]. Moreover, the notion that cytosomes are more frequently heterogeneous in NCL variants, unrelated to primary lysosomal enzyme deficiency, is consistent with the evidence that mixed storage products are readily detectable in skin elements [50]. Further advantages come from the diagnostic investigation of skin. It may eventually be possible to establish skin cell lines to support the identification of still missing NCL genes, for studies on pathogenesis from localization and function of the mutated protein, or even for pharmacologic assays when the time approaches for possible specific treatments.

Nowadays, skeletal muscle is not used for ultrastructural examination in NCL because it has become evident that specific cytosomes are not so easily detectable when compared to other biopsied tissues. Skeletal muscle biopsies, primarily employed in the diagnosis of myopathies, are routinely utilised in the diagnosis of certain progressive encephalopathies, when possible mitochondrial involvement is taken into account. However, when this procedure is used for the differential diagnosis of neurodegenerative disorders (of childhood as well as of adulthood), a search for autofluorescent material and storage accumulation should always be included because features consistent with the diagnosis of NCL might be observed. To take the best advantage of this procedure, therefore, samples frozen as well as embedded in resin, should always be collected from the biopsied specimens. To facilitate diagnostic interpretation in biopsied NCL extracerebral tissues, a list of ultrastructural features that may be encountered in a large variety of non-NCL conditions is listed in Table 1.

4.3. Childhood forms of NCL

4.3.1. CLN1

Mutations in *CLN1*, the gene encoding the enzyme PPT1, cause classic CLN1 disease also known as infantile NCL [48,51]. Typically the onset of the disease begins in infancy but as a result of different mutations in the *CLN1* gene, to date 49 different mutations have been described, it is now recognised that the clinical course and age

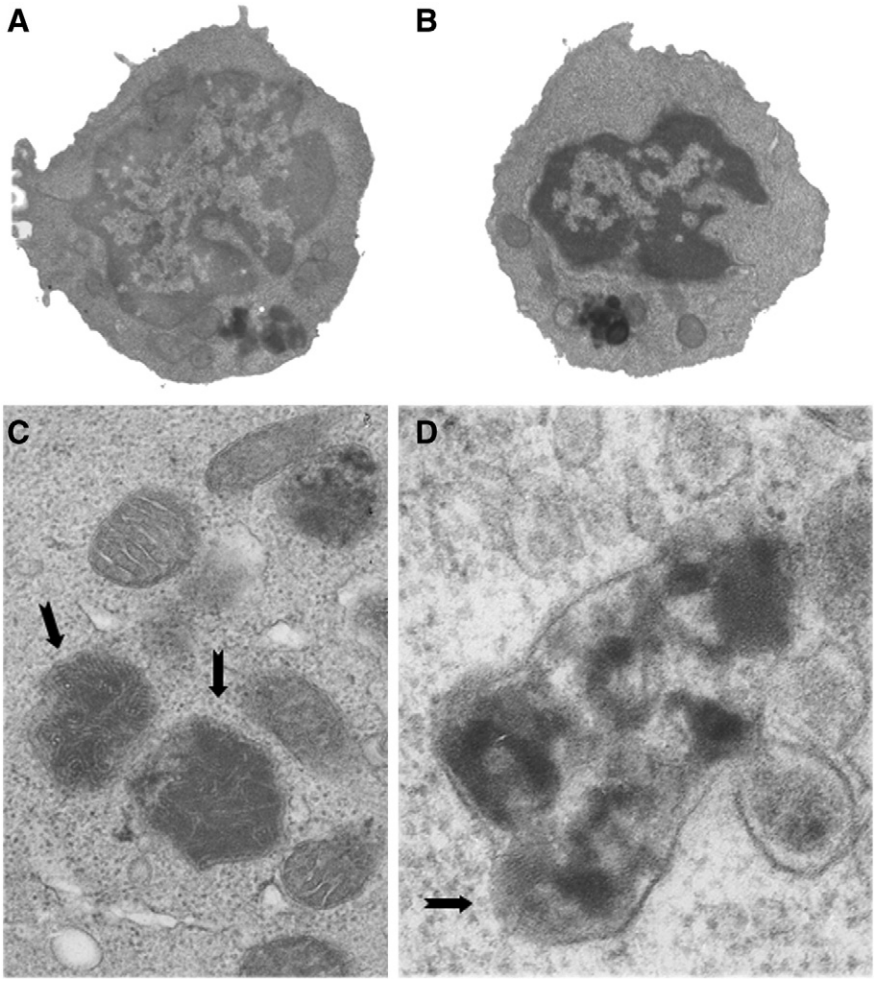

Fig. 1. *EM features of circulating lymphocytes in vLINCL patients*: Similar cytoplasmic inclusions made up by granular and condensed osmiophilic inclusions in CLN5 (A, ×18,000) and CLN8 (B, ×18,000) patients. FPP cytosomes (arrows) in a patient mutated in *CLN7* (C, ×53,000). Mixed osmiophilic inclusions containing FPPs encircled by a single membrane in a molecularly unidentified NCL patient (*CLNX*; D, ×135,000).

of onset of the disease can vary and may present as late infantile, juvenile and adult forms [52–54].

The diagnosis of CLN1 disorder is based on an assay of enzyme activity (PPT1/TPP1), molecular genetic testing and morphological assessment of biopsy samples. Accumulation of typical storage material occurs in the central nervous system and retina and causes severe degenerative changes which are most severe in the infantile form of CLN1. In other sites storage is evident but destruction of the tissue is not a notable feature. It is from these more convenient sites that biopsy samples are taken and examined, primarily by EM, for diagnostic confirmation of the disorder.

4.3.1.1. Histopathology. Extracerebral storage cytosomes are commonly seen in smooth and striated muscle, secretory epithelial cells especially those forming the eccrine sweat glands of the skin, vascular endothelial and smooth muscle cells, ganglion cells of the enteric nervous system and to a lesser extent circulating activated macrophages and fibroblasts. Samples for investigation have included rectal, conjunctival and skeletal muscle biopsies, but it is now commonly accepted that skin biopsy and/or a blood sample for lymphocyte examination can provide essential diagnostic information with minimal patient distress [55].

4.3.1.1.1. Skin biopsy. Skin contains a diverse group of cell types and is often the tissue of choice for the diagnosis of NCL and some other lysosomal storage disorders. A 3 mm punch biopsy is easily obtained from the inner aspect of the forearm or upper arm. A second sample may also be taken for fibroblast culture or DNA studies. Generally for histopathological examination the sample is processed entirely for ultrastructural assessment as routine LM will not provide conclusive or additional diagnostic information. Ideally the area to be examined should include eccrine sweat glands, blood vessels and small peripheral nerves.

4.3.1.1.2. Blood. Examination of blood lymphocytes by LM and EM is a simple, minimally-invasive procedure to study NCL disease [56]. A blood film can be assessed for vacuolated lymphocytes, normally present only in CLN3 disease and some other lysosomal disorders [57]. The remaining sample is centrifuged to form a buffy coat of cells which is fixed in glutaraldehyde and processed into resin for ultrastructural examination.

4.3.1.1.3. Rectal biopsy. Rectal biopsy can be used for the assessment of NCL disease and other neurodegenerative disorders of childhood, however, it is now not the first choice of biopsy. It contains a diverse range of cells including neurons and nerves of the submucosal and myenteric plexuses which may reflect some of the pathological changes that are taking place in the CNS. Unfortunately suitable neurons are not always present in the samples. Rectal tissue has also been used for frozen sectioning which allows for enzyme histochemical analysis and lipid demonstration.

4.3.1.1.4. Other tissue sites. Skeletal muscle biopsy has limited value in the primary assessment of NCL disease and ultrastructural diagnosis may be difficult to interpret as some storage inclusions such as FPPs have not been described.

Table 1
Electron microscopic structures, differential diagnostic to neuronal-ceroid lipofuscinosis (NCL) lipopigments.

Age-related or wear-&-tear lipofuscin
Lipopigments in vitamin-E deficiency
Residual bodies in other lysosomal diseases
(e.g. curved profiles in Farber disease, lamellar bodies and others)
Curvilinear profiles in chloroquine intoxication
Gall bodies in lymphocytes
Tubular arrays in lymphocytes
Undulating tubules/tubuloreticular profiles in endothelial cells
Pi-granules in Schwann cells
Weibel–Palade bodies in endothelial cells
Inclusions in duct cells of eccrine sweat glands
Inclusions in apocrine sweat gland cells
Granules in granulocytes
Granules in mast cells
Inclusions in melanocytes
Non-specific inclusions in macrophages
Inclusions of congenital myopathies in muscle fibres
Polyglucosan bodies
Fingerprint-profile inclusions in vascular smooth muscle cells
Non-specific dense bodies in terminal axons

Conjunctival and peripheral nerve biopsies are rarely examined for this group of disorders but may have value in other lysosomal or neurodegenerative disorders.

4.3.1.2. Histochemistry and fluorescence microscopy. The storage material is generally robust enough to survive routine fixation and paraffin processing. The storage material shows a strong yellowish autofluorescence with ultraviolet light (Fig. 2) but care must be taken to avoid confusion with normal age related lipofuscin which tends to have a more orange autofluorescence. It may be examined by standard histological methods including PAS, LFB with cresyl violet counterstain (Fig. 3) and lipid stains such as Sudan black B (Fig. 4). If frozen section material is available, storage granules are strongly acid phosphatase positive (Fig. 5) [43,58].

4.3.1.3. Immunocytochemistry. Immunocytochemistry in CLN1 disease should include antibodies to SAPs A and D, SCMAS and beta A4 amyloid protein. CLN1 can give a strong reaction for SAPs A and D, the main component of storage material [59], a negative reaction for SCMAS but may react with a monoclonal antibody against beta A4 amyloid protein [60].

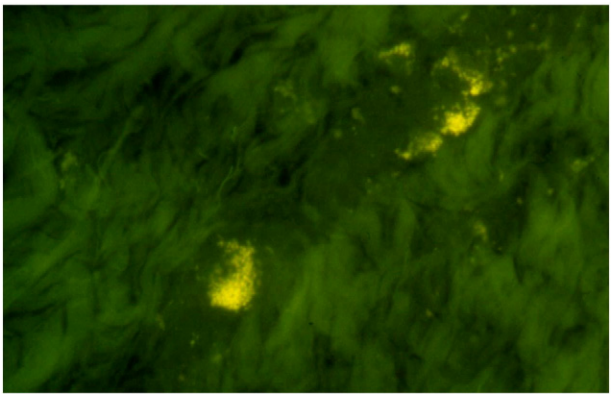

Fig. 2. *Rectal biopsy — general light microscopy (LM) features of CLN disease*: Rectal ganglion cells showing yellowish autofluorescent storage material with ultraviolet light excitation. Unstained paraffin section. ×160.

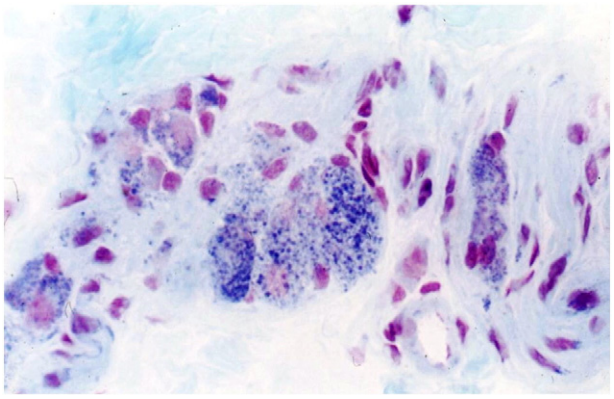

Fig. 3. *Rectal biopsy — general LM features of CLN disease*: Ganglion cells with dark blue cytoplasmic storage granules. LFB ×160.

4.3.1.4. Electron microscopy. The storage cytosomes of CLN1 disease are membrane-bound GRODs. The inclusions vary in size (0.5 μm to 3.0 μm in diameter) and shape and some may have an undulating border with a distinct enclosing membrane [43,58]. The internal granular material can be finely textured and compact, or, coarse and loosely packed showing smaller globular subunits. The electron density of the material may also show some variation and can on occasion be mistaken for other subcellular organelles.

GRODs are seen in various cells of the nervous system including neurons, astrocytes and macrophages in the brain and spinal cord, retinal cells and ganglion cells in the submucosal and myenteric plexus of the bowel.

4.3.1.4.1. Skin biopsy. The skin biopsy shows electron-dense storage material mostly in secretory cells of eccrine sweat glands (Fig. 6) and to a lesser extent in myoepithelial cells. Blood vessels in deeper dermal regions also contain occasional deposits in endothelial (Fig. 7) and smooth muscle cells. Those vessels near the epidermis are often unrewarding. GROD may be present in erector pili muscle, fibroblasts and Schwann cells of the peripheral nerve.

4.3.1.4.2. Blood. Blood samples in CLN1 will demonstrate typical lysosomal storage in 15–20% of lymphocytes in buffy coat preparations (Fig. 8). This low yield may be due to the regular turnover of circulating lymphocytes or possibly a result of the very thin layer of section examined ultrastructurally. It is therefore essential that at least 100 cells are examined to avoid false negative results [56]. Positive cells contain one or two irregular profiles of GROD measuring up to 2 μm at their greatest diameter (Fig. 9). EM also confirms that there are no vacuolated lymphocytes in CLN1.

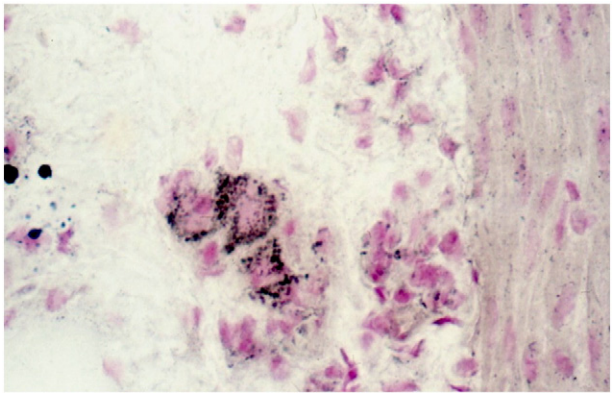

Fig. 4. *Rectal biopsy — general LM features of CLN disease*: Frozen section of rectal biopsy showing lipopigment deposits in submucosal neurons and fine droplets in smooth muscle cells. Sudan black. ×160.

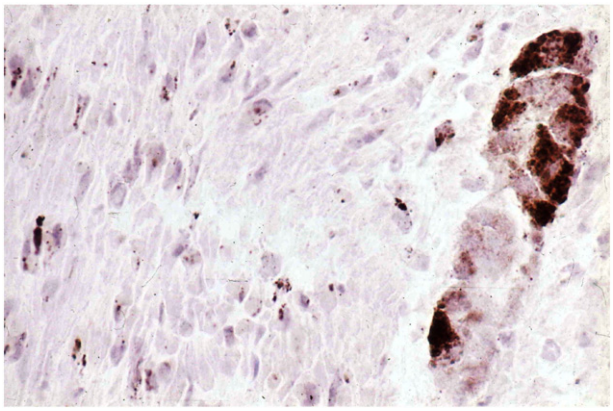

Fig. 5. *Rectal biopsy — general LM features of CLN disease*: Myenteric ganglion cells and smooth muscle cells with positive staining of storage material. Frozen section. Acid phosphatase. ×160.

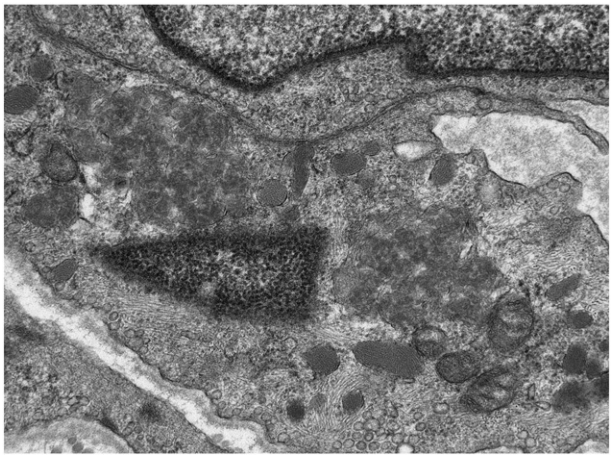

Fig. 7. *CLN1*: Skin biopsy blood vessel endothelial cell with loose texture GROD material. EM ×8000.

4.3.1.4.3. Rectal biopsy. GROD storage can be demonstrated in endothelial and smooth muscle cells of blood vessels, and in the often abundant macrophages of the lamina propria, in rectal mucosal biopsy. Pure storage cytosomes will also be present in neurons but these are generally of low electron density and finely granular in texture (Fig. 10). Smooth muscle cell of the muscularis will display varying amounts of storage material.

4.3.1.5. Prenatal diagnosis. Families with a known risk of CLN1 disease can be offered prenatal diagnosis. A chorionic villus sample (CVS) taken at 12–15 weeks of pregnancy can be examined biochemically for the enzyme deficiency and ultrastructurally for storage inclusions. In CLN1 disease GROD is present in subtrophoblastic blood vessel endothelial cells. Since this can be sparse several vessels should be examined. The cytosomes have a loose texture and are 1 μm or less in diameter (Fig. 11). In a series of CVS examined ultrastructurally [61] this group describes positive findings in CLN1, CLN2, CLN3 and a variant form, CLN6, which has subsequently been confirmed by mutational analysis (Guerreiro et al. 2012, personal communication). It is advisable that if termination of pregnancy follows from a prenatal diagnosis, biochemical and EM examination of foetal tissues should be requested. This is necessary both to provide confirmation and support to the family and for audit of laboratory procedures.

4.3.1.6. CLN1 — late-infantile and juvenile presentation. Several reports have highlighted the later onset of CLN1 type disease caused by mutations in the *CLN1*/*PPT1* gene with characteristic enzyme deficiency and ultrastructural deposition of GROD.

Ultrastructural features in these variant disorders include the familiar storage cytosomes containing GROD. Extracerebral tissues have similar characteristics and storage distribution to that seen in CLN1. There are no vacuolated lymphocytes in the juvenile presentation and GROD is the expected storage inclusion identified in lymphocytes. One group, however, has reported a case showing lymphocytes with GROD combined with CVPs or FPPs [62]. Examination of neurons from rectal biopsies has confirmed the granular nature of the storage cytosomes. In addition an association with small lipid or lipoprotein droplets is also described. This feature is similar to the deposits seen in other examples of variant NCL and dismissed by some workers as normal lipofuscin.

4.3.2. CLN2

Classic LINCL typically has an onset between the ages of two and four years of age. It is characterised by mutations in the *CLN2* or *TPP1* gene, which is located on chromosome 11p15 [63], resulting in decreased lysosomal activity of TPP1. Many other forms of NCL have a late-infancy onset including CLN5, CLN6, CLN7 and CLN8. These are known collectively as vLINCL and are caused by other gene mutations. Typically diagnostic assessment of CLN2 disease should include enzyme assay (TPP1), molecular genetics and morphological assessment. The choice of extracerebral biopsy has been discussed earlier and applies to CLN2 disease as well.

4.3.2.1. Histopathology. Extracerebral tissues show no obvious histopathological changes by LM but storage cytosomes are present and

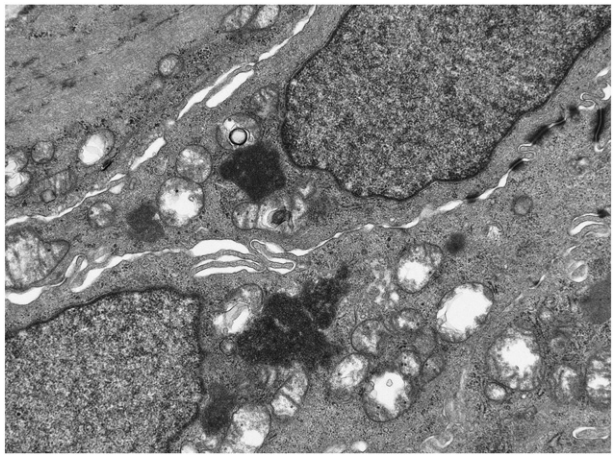

Fig. 6. *CLN1*: Skin biopsy eccrine sweat gland epithelial cells with electron dense storage deposits. EM ×5000.

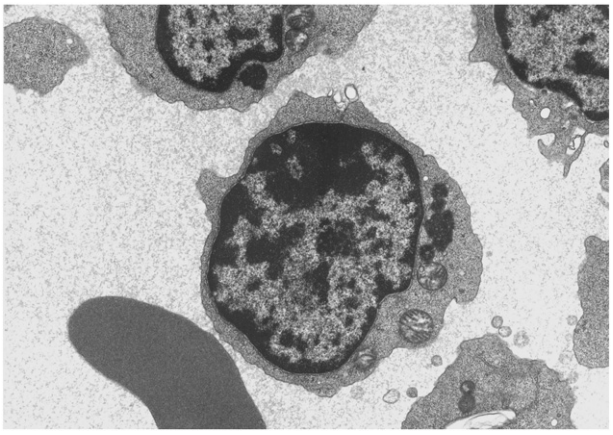

Fig. 8. *CLN1*: Buffy coat lymphocytes with irregular GROD cytosomes. EM ×3000.

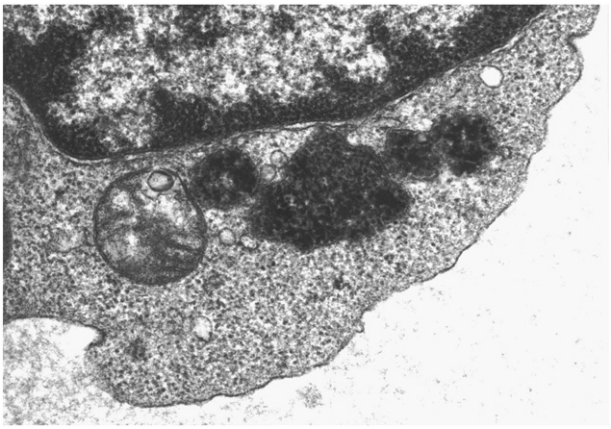

Fig. 9. *CLN1*: Lymphocyte with membrane bound GROD deposits. EM ×15,000.

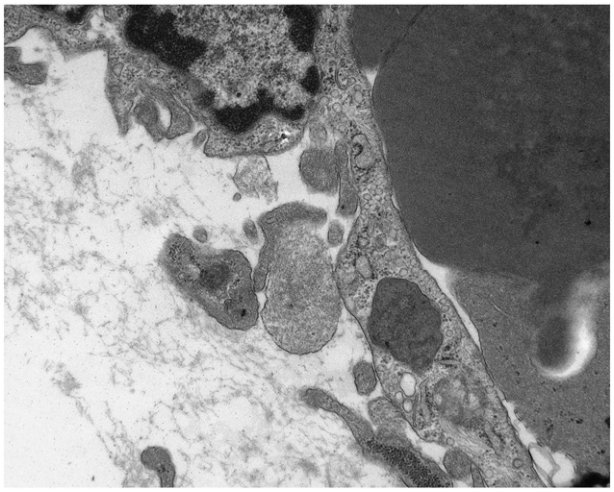

Fig. 11. *CLN1*: CVS showing a single GROD cytosome in a subtrophoblastic blood vessel endothelial cell. EM ×8000.

have comparable staining reactions as described previously. Classically storage is present and identified in rectal biopsy neurons which may have a foamy appearance on haematoxylin and eosin staining. Smooth muscle and endothelial cells also display punctate staining indicating discrete storage inclusions that exhibit strong autofluorescence. Eccrine sweat glands of skin appear normal by routine LM but will display evidence of positive staining for storage inclusions. This, however, cannot be distinguished from the normal staining reaction of secretory cells present in the area. Storage has also been identified in various endocrine tissues including the endocrine and exocrine cells of the pancreas.

4.3.2.2. Immunocytochemistry. Storage material in CLN2 disease shows strong staining of SCMAS [64]. Immunoreactivity is preserved and can be demonstrated in routine paraffin-processed material allowing archival review of stored material [65]. Increased reactivity for SAPs A and D has also been documented with some inconsistent amyloid precursor protein reactivity [60] but there is no evidence of ubiquitin reactivity.

4.3.2.3. Electron microscopy. Classic CLN2 disease shows pure CVPs in all cells involved in the disease process. CVPs are uniformly curved short thin lamellar stacks of alternating dark and light lines with minimal variation in their overall shape. The lamellar profiles are surrounded by a single membrane, confirming the lysosomal nature of the disorder. The CL profiles are in an electron lucent matrix and the enclosed structure forms a CVP body. CL storage in CLN2 disease is not associated with other storage bodies or lipofuscin. The only exception to this is the occasional fragmentary fingerprint stacks that may be seen in CL cytosomes in lymphocytes in CLN2 disease.

4.3.2.3.1. Skin biopsy. The presence of numerous pure CVP bodies in secretory epithelial cells of eccrine sweat glands (Fig. 12), makes skin biopsy a preferred sample type for analysis. CVP inclusions are also often located in endothelial and smooth muscle cells of blood vessels (Fig. 13). Myelinated and small non-myelinated nerve fibres, surrounding sweat glands and blood vessels, occasionally demonstrate storage cytosomes. Fibroblasts may also have CVP inclusions in the cell body.

4.3.2.3.2. Blood. Blood buffy coat lymphocytes are not vacuolated in CLN2 disease. Approximately 15–25% of lymphocytes show one or two, irregular, membrane-bound CVP bodies (Fig. 14) of low electron density with an indistinct fingerprint stack (Fig. 15). For these reasons examination of at least 100 cells is normally recommended.

4.3.2.4. Prenatal diagnosis. Prenatal diagnosis is generally provided by enzyme assay and mutational analysis [66–68]. However EM of CVS biopsy obtained at 12–15 weeks gestation can provide rapid, additional support. In CLN2 disease, discrete CVP inclusions have been identified in subtrophoblastic blood vessel endothelial cells [61]. The larger blood vessels are more productive for identifying these CVP inclusions (Fig. 16). If termination of pregnancy follows a prenatal diagnosis, biochemical assay and EM testing should be requested from foetal tissues for confirmation [69].

4.3.3. CLN3

JNCL or CLN3, the classical juvenile form of NCL is the most common disease variant, at least in Northern Europe, and has a worldwide

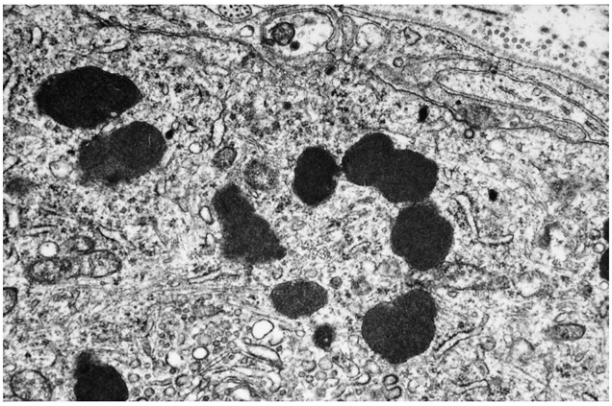

Fig. 10. *CLN1*: Rectal biopsy neuron showing finely granular storage inclusions. EM ×12,000.

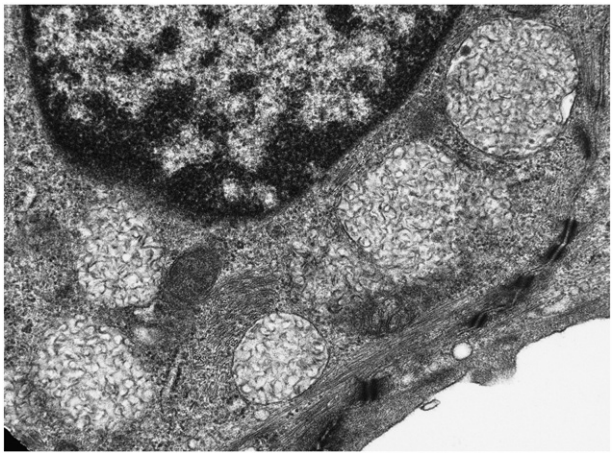

Fig. 12. *CLN2*: Skin biopsy epithelial cell with several pure membrane bound curvilinear profiles. EM ×8000.

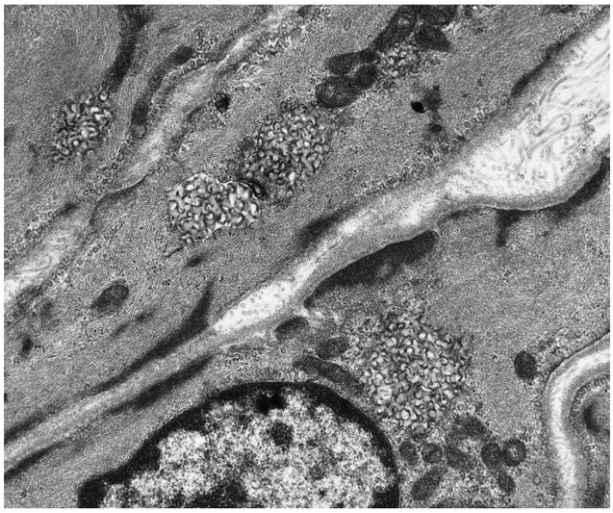

Fig. 13. *CLN2*: Dermal vascular smooth muscle cells with curvilinear profiles. EM ×5000.

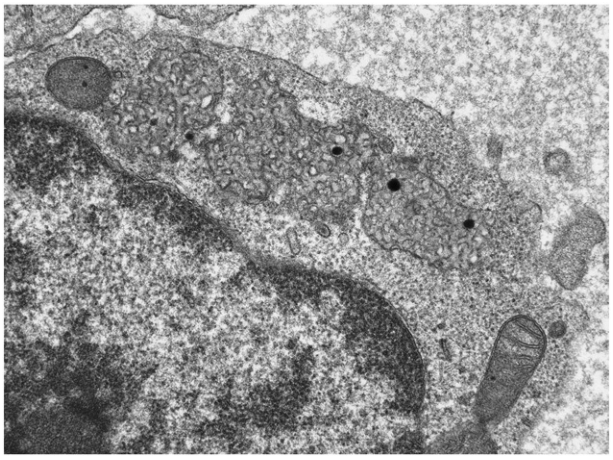

Fig. 15. *CLN2*: Curvilinear profiles with fragments of dense deposits. EM ×15,000.

distribution [70]. It is due to mutations of the *CLN3* gene (the most common being a 1.02 kb homozygous deletion), which encodes a membrane protein pCLN3, predicted to have six transmembrane domains spanning either the endoluminal or the cytosolic domains. The protein is expressed ubiquitously and has been localised mostly in the endosomal/lysosomal compartment, but is also recognised in the synaptosomal fraction (in neuronal cells) and, to a lesser extent, in the plasma membrane. There is evidence that any or all of a number of cell functions, including pH maintenance and osmoregulation, membrane trafficking, cell signalling, etc., are disrupted by mutations in the *CLN3* gene [71]. These exert negative effects on CNS cells specifically, leading to selective cell death of neuronal subpopulations. Clinical onset is by school age (5–8 years), visual failure being the main presenting symptom in the vast majority of cases. Learning difficulties and behavioural disturbances usually follow, whilst motor symptoms, mental deterioration and seizures are relatively delayed. The disease is definitively progressive, showing, however, different patterns of evolution [72]. Death occurs during the late third/early fourth decade in patients carrying the most common homozygous deletion in the *CLN3* gene, whereas longer survival is expected for compound heterozygous patients. Cardiac involvement due to storage accumulation, which affects the conduction system, is relatively common and may lead to fatal arrhythmia; heart rhythm disturbances can, however, benefit from pacemaker fitting [73].

4.3.3.1. Histopathology. Pathological features of extracerebral tissues in classical JNCL/CLN3 have been of diagnostic use for decades, since vacuolated lymphocytes were first observed in blood smears stained with modified Giemsa technique [74].

Enhanced histochemical activity of acid phosphatase, and presence of Sudan black positive material and of autofluorescence can be detected in some cell populations in skin biopsy (endothelial cells, sweat gland epithelia, smooth muscle cells), in skeletal muscle cells, and in ganglionic neurons (and surrounding cellular elements) following rectal biopsy. Most of these cells are immunoreactive to antibodies against SCMAS. Vacuolated cells can be better visualised in semithin sections of biopsy samples or of buffy coat embedded in resin, particularly lymphocytes and ganglionic neurons. Vacuoles can look empty, or some solid material can be identified within them.

4.3.3.2. Electron microscopy

4.3.3.2.1. Blood. Ultrastructural analysis of blood lymphocytes is a reliable method to assess probable CLN3 patients. Either membrane-bound empty vacuoles, or vacuoles containing dense material (usually FPP at higher magnification) can be detected in at least 15–20% of cells, even at the earliest stages of the disease; furthermore, isolated FPPs can be detected in the cytoplasm (Fig. 17).

4.3.3.2.2. Other extracerebral tissues. Membrane-bound vacuoles and cytosomes can be found in several other extracerebral tissues,

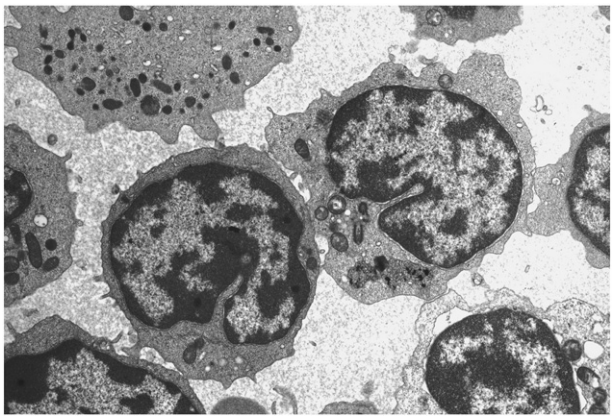

Fig. 14. *CLN2*: Lymphocyte with three curvilinear profiles. EM ×5000.

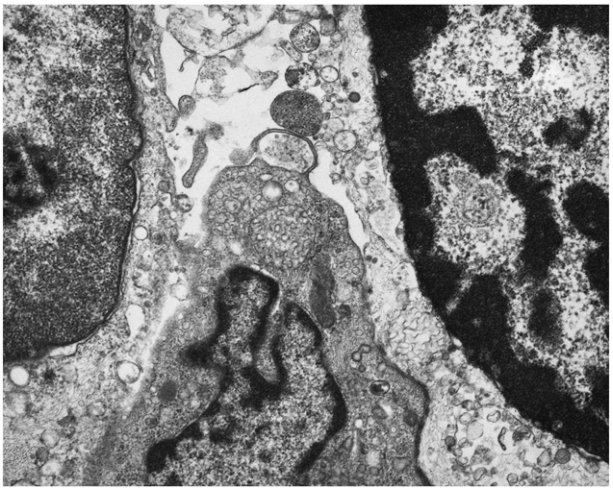

Fig. 16. *CLN2*: CVS endothelial cell with rudimentary membrane bound curvilinear profiles. EM ×10,000.

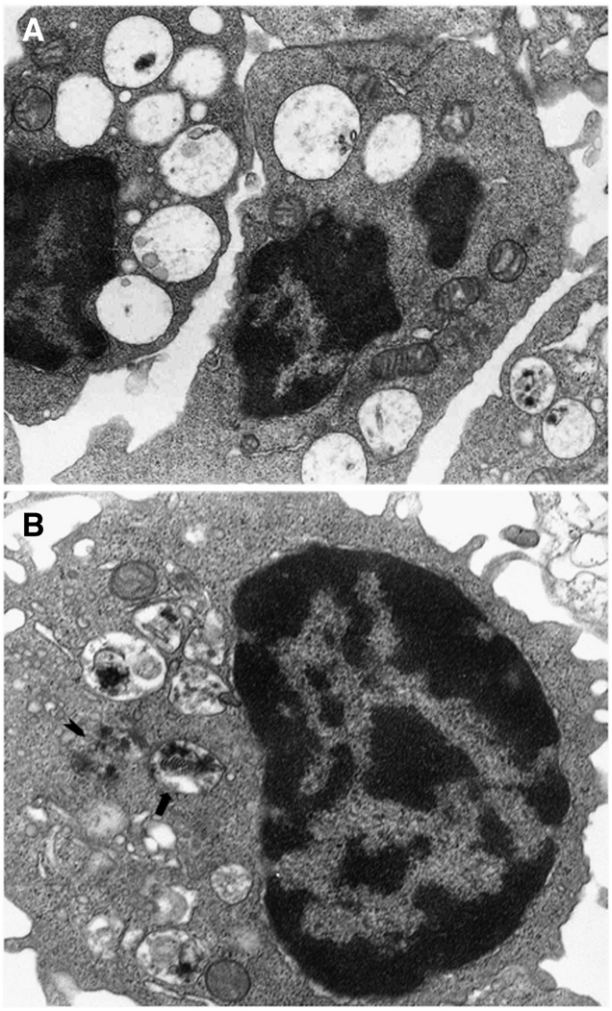

Fig. 17. *CLN3 — blood lymphocytes*: Ultrastructural features of lymphocytic vacuoles. Multiple empty vacuoles of large size (A, ×23,500); smaller vacuoles containing osmiophilic material of different features, including FPP (arrow); note free FPP in the cytoplasm (arrowhead; B, ×37,000).

including the skin, skeletal muscle and rectal mucosa, showing some tissue specificities (Fig. 18). In skeletal muscle, storage within the fibres commonly appears as RLP, whereas mixed FPP/RLP inclusions can be observed in endothelial cells. In rectal mucosa, a mixed population of cytosomes is also detected, along with FPP (within or outside vacuoles), packed concentric membranous cytoplasmic bodies can be seen, featuring the same pattern as observed in gangliosidoses. The presence of mixed inclusions (sometimes including CVP) can also be present in skin elements.

4.3.3.3. Prenatal diagnosis. Prenatal diagnosis was reported by ultrastructural analysis of chorionic villi (as well as aborted foetuses), though diagnosis was difficult based on stored material. In fact, following a unique report showing FPP in the chorionic villi at 9th week of gestation [74], inclusions containing lamellar material and membranous fragments, but neither CVP nor FPP, were detected [75]. Such findings may represent the early stages of aggregation of storage material before classical cytosomes are formed. This procedure has been replaced by molecular testing in at-risk pregnancies and for suspected carriers.

4.3.3.4. Pathogenetic considerations. The patho-morphology of CLN3 disease is quite constant and no meaningful variations are detected according to either the patterns of disease course or the kind of mutations. The presence in several tissues of large vacuoles containing storage material is consistent with activation of the autophago-lysosomal pathway, and ultrastructural evidence of early autophagosome formation can be detected even in biopsy material (AS, personal observations). The relevance of this phenomenon in CLN3 (as compared to other CLN diseases), indicates that autophagy is a major cellular event in this human disease [76]. Whether disturbances of this process also play a negative role in CNS neuron survival during the disease has not yet been demonstrated. However, it was taken into account in the debate over the mechanism of neuronal cell death in CLN3, since no evidence of other cell-death patterns (such as apoptosis) has been demonstrated in CLN3-deficient mice. Rather, these show remarkable vacuolization of the neuronal cytoplasm, consistent with impaired autophagosome maturation and clearance [77,78].

4.3.4. CLN5

CLN5 disease is a rare vLINCL, first described in Finland, and later identified in several countries worldwide [7]. It occurs due to mutations in the *CLN5* gene, which codes for a soluble lysosomal protein, whose function is not yet precisely known. It has recently been suggested that it may play a role in controlling trafficking from the endoplasmic reticulum, (ER) through the Golgi apparatus, to the lysosomal compartment [79]. Clinical onset of CLN5 may be between 5 and 7 years of age and is late compared to other late-infantile forms. It features either signs of psychomotor regression or early behavioural abnormalities, and early speech disturbances. Seizures occur in all cases but are not necessarily an early symptom.

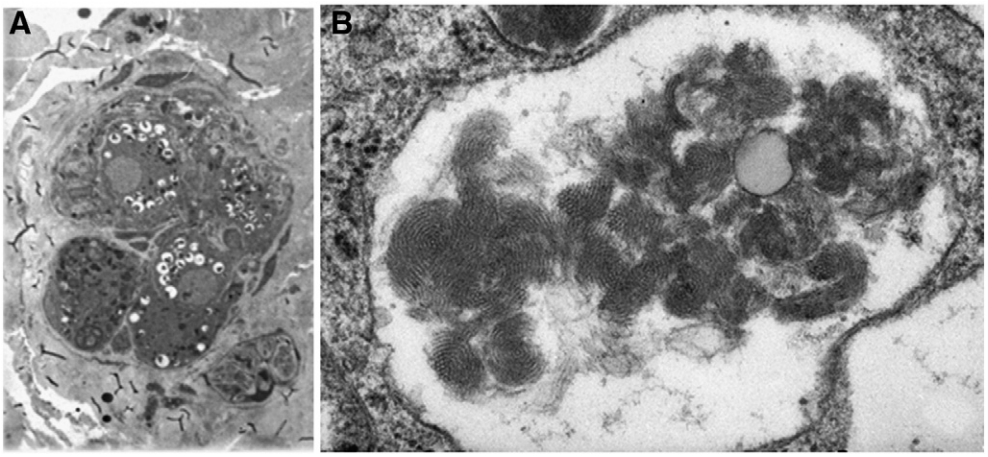

Fig. 18. *CLN3 — rectal biopsy*: Vacuolated cytoplasm of ganglionic neurons (A, ×900); single membrane-limited vacuoles filled with FPP, showing prevalent semicircular orientation (B, ×113,000).

4.3.4.1. Electron microscopy. CLN5's clinical heterogeneity makes a straightforward approach towards molecular diagnosis difficult and, therefore, ultrastructural analysis of extracerebral tissues is commonly performed first, in order to recognise the presence (or absence) of storage material. A mixed population of CVP and FPP lysosomal inclusions are detected in skin cells in this condition. Either cytosomes can be observed singly, affecting different cells of the same patient, or they may occur as a complex structure containing both, CVP and FPP. RLP and condensed osmiophilic material can also be present in this condition. Blood lymphocytes show lysosomal storage with nonspecific features, such as granular material and condensed osmiophilic globules, commonly embedded in a homogeneous matrix; FPPs can also be identified (Fig. 19). Single cytosome storage (namely of FPP) is rare but it has been reported in two patients carrying different mutations affected with early-juvenile and juvenile phenotypes [80,81]. Autophagic vacuoles were also described, which may suggest that this process activates a possible rescue mechanism of the cell to get rid of the abnormal storage [82].

4.3.4.2. Prenatal diagnosis. Mixed cytosomes, featuring either CVP or FPP, were reported in chorionic villi (pericytes and endothelial cells) at 14th week's gestation [83] but molecular testing has largely replaced EM analysis for prenatal diagnosis of CLN5.

Genotype–morphotype correlations are difficult, due to the relatively small number of reported patients. However, the kind of mutation (either missense or nonsense) does not correlate with age of onset, clinical severity, nor the quality of storage population; conversely, the clinical severity does seem to be partly related to the quantity of mixed storage observed in the examined tissue.

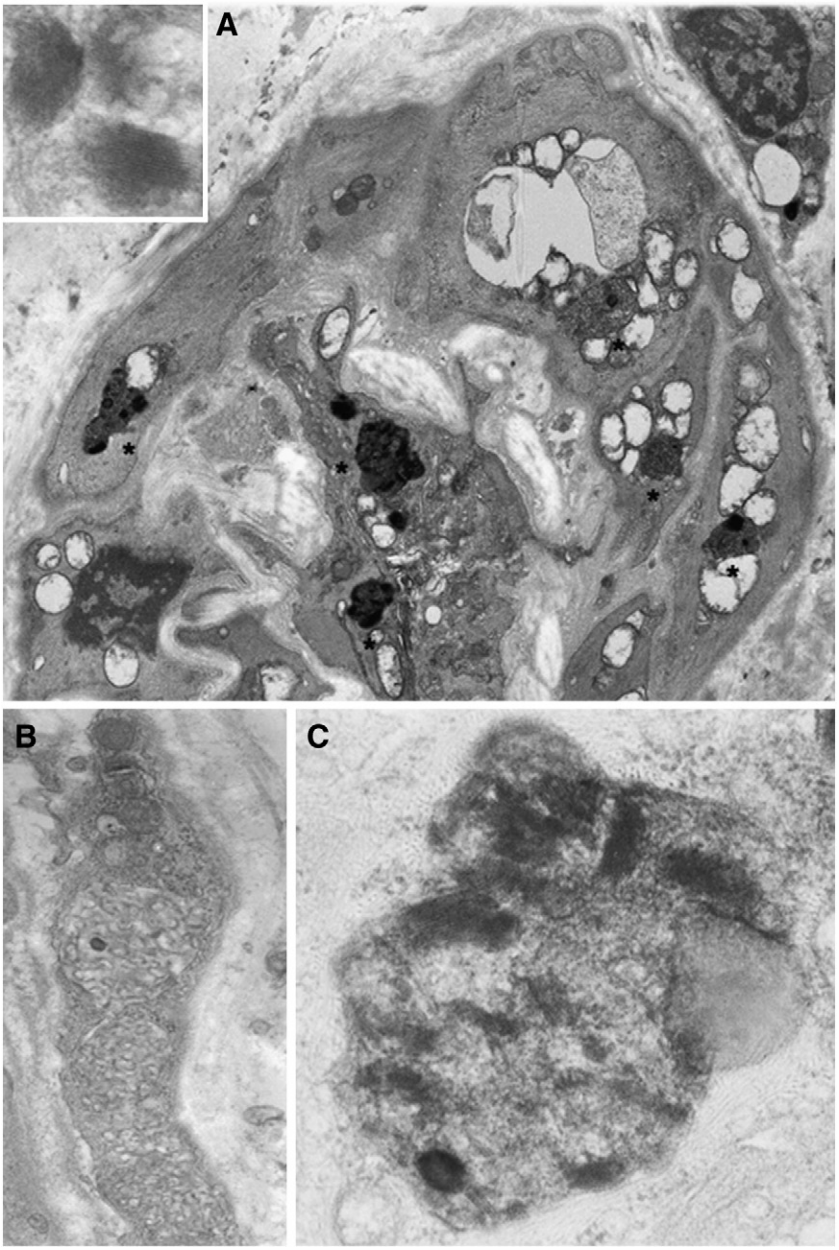

Fig. 19. *CLN5 — skin biopsy*: Osmiophilic inclusions scattered in the cytoplasm of different cells (asterisks); some of the inclusions are made up of FPP (see inset, ×72,000; A, ×11,500); CVP outlined by a single membrane filling a fibrocytic process (B, ×34,500); mixed population with a prevalent FPP component (asterisks) embedded in a loose matrix (C, ×70,500).

4.3.5. CLN6

Mutations affecting the *CLN6* gene are related to a wide phenotypic spectrum as far as age of onset and disease duration are concerned. *CLN6* gene mutations can give rise to variant LINCL, to early JNCL, as well as to ANCL (Kufs A type) [4,15]. The *CLN6* gene encodes a conserved, transmembrane polytopic protein of the ER (pCLN6) whose function has yet to be identified. The relevance of the autophagolysosomal compartment observed in human fibroblasts, however, suggests that mutated pCLN6 may hamper lysosomal function. Mutations in the *CLN6* gene have worldwide distribution, with elevated frequency in Southern European countries compared to those further North [84].

The clinical features of CLN6 disease of childhood onset are similar to classical LINCL, and to vLINCL associated with mutations in the *CLN7* or *CLN8* genes; with generalised seizures and motor symptoms. The rate of disease progression may vary to some extent, with a relatively slow and more protracted course, but the disease symptoms are similar to the other forms: motor impairment, myoclonic seizures, speech degradation, visual loss, and mental deterioration, leading to severe disability in mid-adolescence and death by the end of the second decade in life. In CLN6-related vLINCL both electroretinogram (ERG; response extinction) and evoked potentials (EP: giant cortical EP) are abnormal [50].

4.3.5.1. Electron microscopy. Ultrastructural analysis of skin biopsy or blood lymphocytes is useful, particularly during the early stages of the disease. The cytosome pattern helps to discriminate between cLINCL and vLINCL and determines, therefore, which biochemical pathways should be tested and/or genes sequenced. The storage population in CLN6 disease is mixed and pleomorphic: CVP and FPP are the major components with RLP, condensed osmiophilic bodies, and granular material less frequently seen. In skin biopsy, cytosomes can be detected in any cell type, either as lysosomal structures or, less frequently, free in the cytoplasm (Fig. 20). Smooth muscle cells can be affected, but less heavily than in CLN2 disease. Vacuoles can be detected, either empty or containing osmiophilic material of varying patterns, and dilated cisternae of the ER are present. Cytoplasmic inclusions such as granular or condensed bodies, or even RLP, can be seen in blood lymphocytes.

4.3.5.2. Prenatal diagnosis. A recent report of ultrastructural findings in a CVS in CLN6 found storage inclusions, in subtrophoblastic blood vessel endothelial cells, that are amorphous and of medium electron density with occasional stacked lamellae. Subsequently, storage material was confirmed in the aborted foetus and the diagnosis of CLN6 established by mutation analysis (Guerreiro et al. 2012, personal communication).

4.3.5.3. Pathogenetic considerations. The evidence of large vacuoles and autophagosome-like structures is consistent with impaired functioning of the autophagic system in this condition [50]. At present there is no evidence of genotype–phenotype correlations as far as the clinical onset and disease course in CLN6 disease are concerned; likewise, the reported mutations do not allow any genotype–morphotype relationship since the amount of storage and the relative heterogeneity of the inclusions seem to be similar in all the reported cases.

4.3.6. CLN7

A severe vLINCL form is related to mutations in the *CLN7* gene, which codes for a transmembrane protein of the lysosomal membrane, where it functions as a transporter, the substrate specificity of which is still unknown [8]. Recently identified in Turkish kindreds [85,86], it has also been described in patients of various ethnic origins, but its frequency seems to be higher among many Mediterranean populations [87]. As mentioned before, clinical presentation at onset is similar to other LINCL, such as CLN2, CLN6 and CLN8. Generalised seizures and developmental regression are present at onset, followed by myoclonus, motor impairment with ataxia, speech and mental deterioration, visual failure and characteristic behavioural and sleep disturbances. The rate of progression is quite steady and the outcome fatal within the second decade. Intrafamilial heterogeneity can be present. Early involvement of the visual pathways is associated with specific neurophysiological findings: occipital spikes during sleep and possible photic response at EEG, giant occipital response following visual EP, and ERG extinction.

4.3.6.1. Electron microscopy. Since the presenting clinical features of CLN7 and other LINCLs are so similar, an ultrastructural approach can indicate which biochemical and/or genetic assessment should follow to achieve a differential diagnosis. Findings from skin, muscle and rectal biopsies as well as blood lymphocytes have been reported in CLN7 disease (Fig. 21). FPPs are the most frequently represented cytosomes, seen in all of these tissues. They can be detected as single storage bodies, or forming a mixed inclusion with either irregular CVP, or RLP. Less evident than in other vLINCL is the presence of

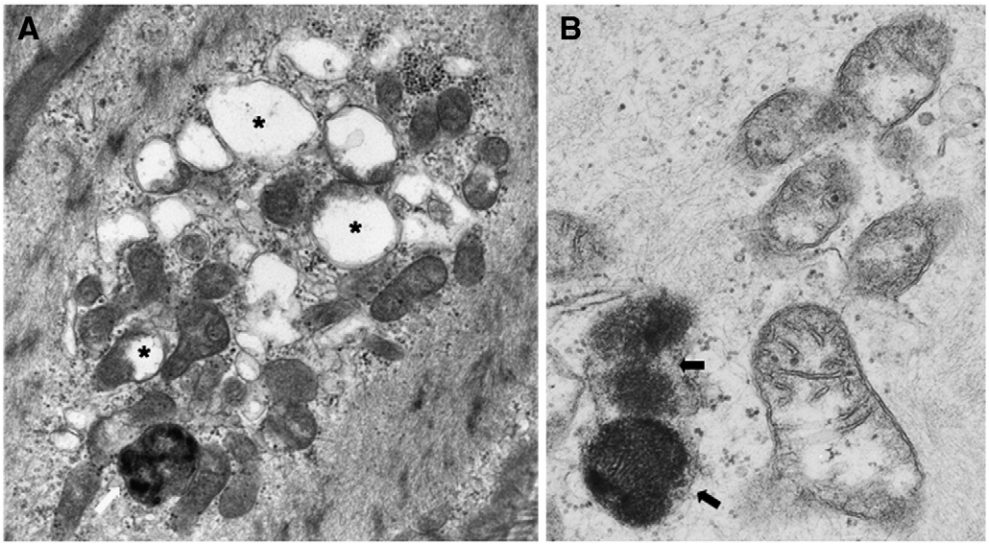

Fig. 20. *CLN6 — skin biopsy*: Double membrane vacuoles featuring autophagosome-like figures (asterisks); note a cytosome characterised by osmiophilic material of different size and conformation embedded in finely granular matrix and outlined by a single membrane (arrow; A, ×34,000); aggregated tubular profiles and FPP apparently free in the cytoplasm of a smooth muscle cell (arrows; B, ×58,000).

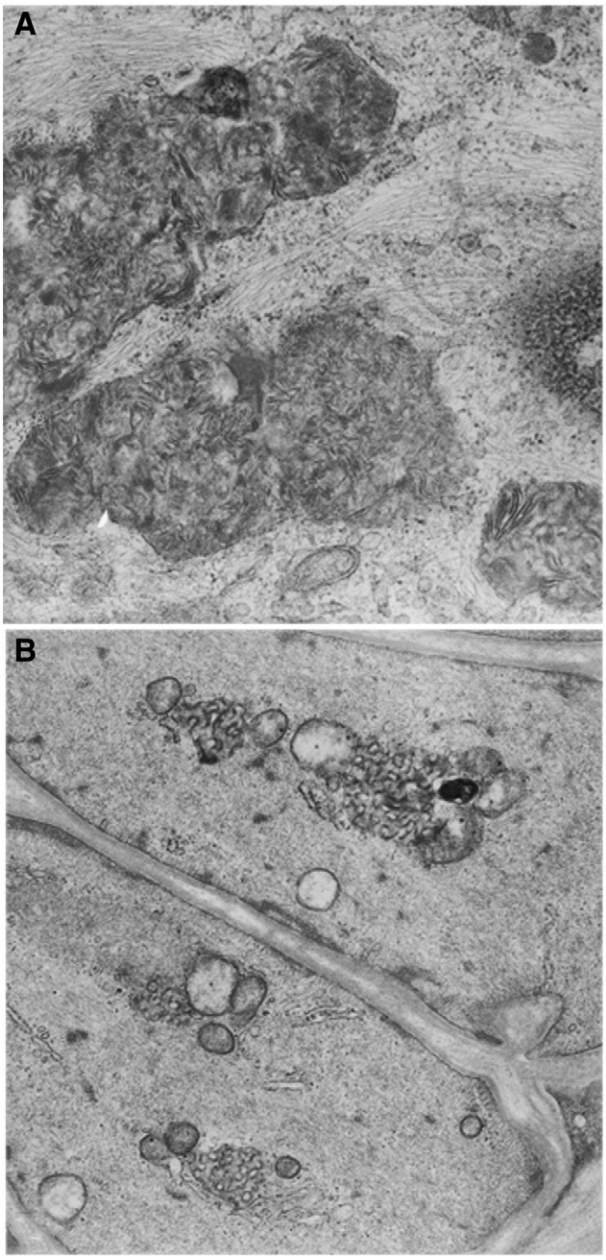

Fig. 21. *CLN7 — skin biopsy, smooth muscle cells*: Mixed inclusions (RLP and FPP) embedded in a dense matrix (A ×40,000) and CVP cytosomes (B, ×23,000).

nonspecific granular inclusions. CVPs never predominate in CLN 7 as they do in cLINCL. Vacuolated lymphocytes are extremely rare and, when present, the number of vacuoles in lymphocytes is remarkably small.

4.3.6.2. Prenatal diagnosis. To date, no ultrastructural studies report abnormalities in chorionic villi or foetal tissues.

Ultrastructural pathology is not suggestive of any abnormal cellular process occurring due to *CLN7* gene mutations. Both clinical features and patterns of ultrastructural pathology are relatively homogeneous in CLN7 disease and, therefore, there is no firm genotype–phenotype correlation. Likewise, the patho-phenotype does not seem to be affected by this kind of mutation.

4.3.7. CLN8

Two different NCLs with different geographical distribution and clinical evolution are grouped together under the term CLN8 disease [2]. The mutated gene, *CLN8*, encodes a transmembrane protein of the ER, which seems to function as a sphingolipid sensor and may be involved in glycosphingolipid trafficking [88]. CLN8 was first described in Finland as progressive epilepsy with mental retardation (EPMR), also known as Northern epilepsy syndrome. It is characterised by late-infantile onset with epilepsy and cognitive decline, followed by relative clinical stability and slow progression into adulthood. A genetic marker, a single homozygous mutation in the *CLN8* gene, has been identified in the Finnish population. A panel of different mutations in the *CLN8* gene was later detected in a Turkish cohort of children also affected with a late-infantile-onset disease. This allelic LINCL variant was recognised to have a worldwide distribution [85,89]. In vLINCL CLN8, the age of onset can be quite early (within the second year of life), with seizures and progressive ataxia, followed by psychomotor regression and myoclonus. The visual system with retinal involvement is always affected, with ERG extinction. Severe involvement of cortical structures is outlined by the giant cortical potential elicited by EP. The outcome is fatal by the end of the second decade.

4.3.7.1. Electron microscopy. Storage accumulation is characterised by cytosome heterogeneity, detectable in different tissues (skin, lymphocytes, rectal mucosa), where a mixed CVP-FPP population is observed within the lysosomes, commonly embedded in either a loose or granular matrix. Both CVP and FPP can be detected separately, or may form a single cytosome which contains both components. FPP can be observed with classical membrane periodicity, condensed as osmiophilic globules, or may show a loose pattern [90,91]. Other storage material may be present, such as osmiophilic granules (particularly in the lymphocytes) and RLP (Fig. 22). Such pathological heterogeneity is seen in both NCL forms associated with *CLN8* mutations; vLINCL and EPMR. No vacuolated lymphocytes are detected.

4.3.7.2. Prenatal diagnosis. Prenatal diagnosis by ultrastructural investigation has never been described.

A clinico-genetic correlation may be possible; there is evidence that the most severe cases are associated with nonsense mutations (leading to either no transcripts, truncated protein, or affecting a conserved domain of pCLN8), but there is no clear relationship between the pathological features (in terms of amounts of cytosomes, or ultrastructural complexity) and the genotype.

4.3.8. CLN9

A small number of patients affected with a disease course similar to classical JNCL, but without any known *CLN3* gene mutation, have been classified as "CLN9 type". This rare form is associated with abnormalities in sphingolipid metabolism (in fibroblasts), as well as with decreased activity of dihydroceramide synthase. The mutated gene associated with this condition remains unknown [92]. Vacuolated lymphocytes have been described, either empty, or containing electron-dense storage with FPP pattern, but findings from other peripheral tissues have not been reported.

4.3.9. CLN10

CLN10 disease begins in utero, and this congenital disorder is a rare and severe form in the spectrum of NCL disease which was first described in the early 1940s [93]. Its transmission is autosomal recessive and it is caused by mutations in the *CTSD* gene (designated *CLN10*; 11p15.5) encoding the lysosomal enzyme cathepsin D [94,95]. It is characterised by poor intrauterine foetal growth rate, microcephaly and seizures [94]. Although the disease is rare many cases may be undiagnosed as affected foetuses may be non-viable and are probably spontaneously aborted or stillborn. There is no effective treatment for congenital NCL and survival is a matter of hours or days, rarely longer.

Later onset forms of CLN10 have been reported in the literature [95] and are thought to be due to partial inactivation of the *CTSD* gene. This form of CLN10 has a more prolonged neurodegenerative

course with ataxia, progressive cognitive decline, speech loss and retinal atrophy.

Diagnosis of CLN10 is based on enzymatic testing for CTSD deficiency, granular storage material in tissue cells and molecular confirmation of the *CTSD* gene mutation. CLN1/PPT1 deficiency should be excluded as a possible differential diagnosis.

4.3.9.1. Electron microscopy. Skin biopsy ultrastructure from a patient with late onset CSTD deficiency revealed granular storage material in non-myelinated nerves. No storage material was identified in eccrine sweat glands, or in fibroblasts or endothelial cells [95]. This is an unusual feature as in most other NCL disorders storage cytosomes are present in many cell types.

Examination of a significant number of peripheral blood lymphocytes from the same late onset CSTD patient showed no evidence of storage material [95].

Experimental data suggest that in CSTD deficiency there is ultrastructural degeneration of synaptic junctions leading to an increase in releasable synaptic vesicles. CTSD is required for normal synaptic function, and a failure in synaptic trafficking or recycling may be an early and important pathologic mechanism initiating synaptic degeneration in advance of subsequent neuronal loss [96].

4.3.9.2. Prenatal diagnosis. To date there are no published data on ultrastructural storage in prenatal samples. A novel mutation in the cathepsin D gene, identified in one family, has been used to exclude CSTD deficiency in a younger sibling of the affected patient [97].

4.3.10. CLN12

Four siblings with juvenile onset of NCL were found to have a single homozygous mutation in the *ATP13A2* gene, rendering this newly identified gene the cause of a new NCL form, CLN12. Lymphocytes showed vacuoles and, ultrastructurally, contained NCL-typical lipopigments, which, by biopsy, were also seen in skeletal muscle [21].

4.3.11. CLN14

Siblings from three families from Mexico, Morocco and Turkey with infantile onset (9–24 months of age), progressive myoclonic epilepsy were found to have homozygous mutations in *KCTD7*, a gene which encodes a potassium channel tetramerization domain containing protein 7. Lysosomal storage products (GRODs and RLPs), detected in peripheral blood lymphocytes and skin fibroblasts of one patient, were consistent with the inclusions detected in NCL. Therefore mutations in *KCTD7* can be associated with an infantile onset NCL, named CLN14 [98].

4.3.12. Unidentified NCL/CLNX

In about 10–12% of patients affected with a progressive neurological disorder, whose clinical and neurophysiological features are consistent with a diagnosis of NCL, genetic mutations remain undefined. The age of onset of the disease in these patients is usually within the range of the vLINCL, but later onset cases can also be observed. Ultrastructural analysis of peripheral tissues shows the presence of lipopigments and endolysosomal storage with heterogeneous patterns, but rarely containing classical cytosomes (Fig. 23). The identification of new informative NCL families is therefore necessary in order to recognise new NCL genes.

4.4. Adult forms of NCL

The usefulness of ultrastructural examination of extra-cerebral tissues, as compared to brain biopsy, in ANCL is still debated [45,99]. The

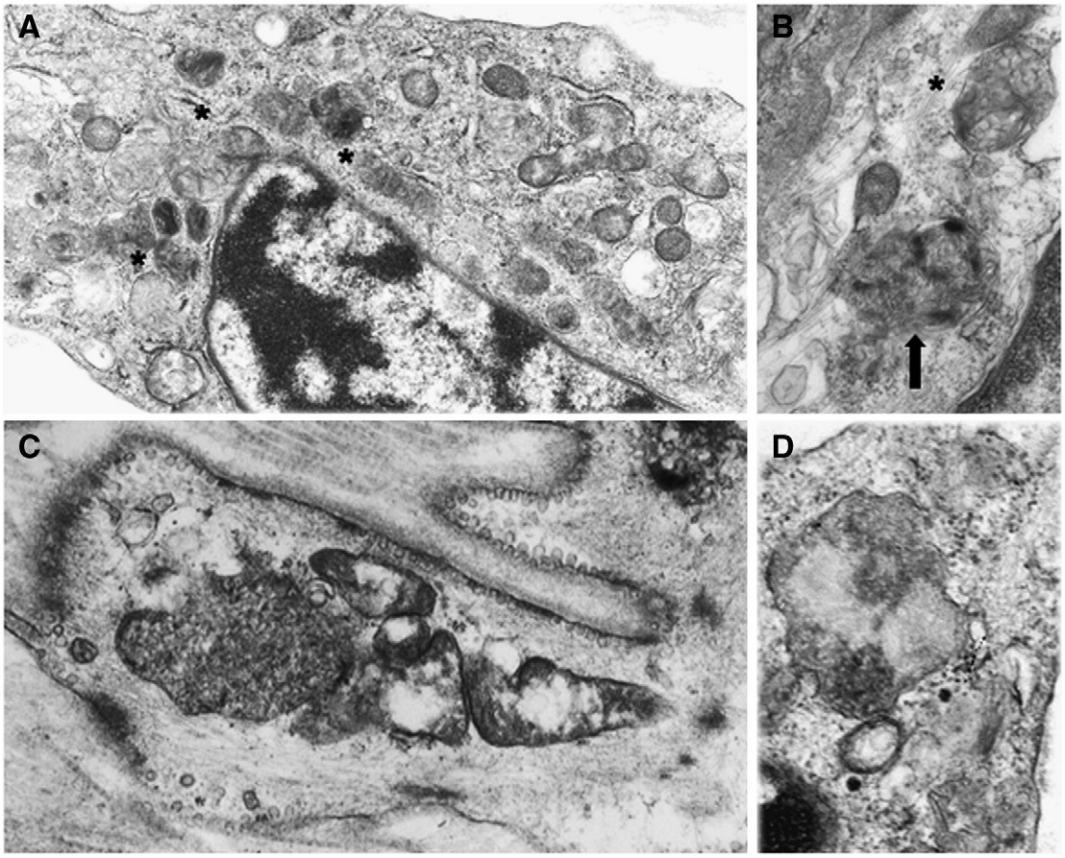

Fig. 22. *CLN8 — skin biopsy*: Fibrocytic processes (A, B, D); smooth muscle cell (C). Heterogeneous membrane-bound storage population: round, osmiophilic inclusions (dark asterisks; A, ×26,000), globular cytosomes with round profiles (dark asterisk) and FPP (arrow; B, ×37,000), elongated cytosome filled with curvilinear-like profiles (C, ×32,000); granular material embedded in a loose matrix (D, ×45,000).

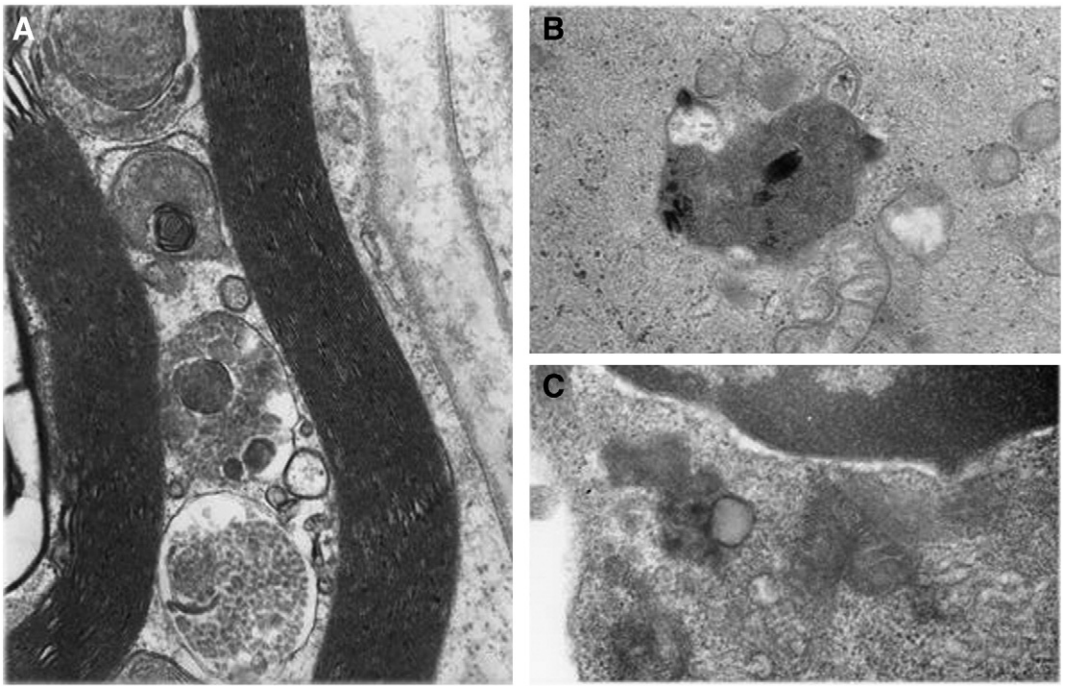

Fig. 23. *CLNX — skin biopsy and blood lymphocyte*: A. Putative mixed cytosome population in the subaxonal space of an intradermal nerve fibre (A, ×38,000); homogeneous body with highly osmiophilic inclusions embedded in a smooth muscle cell (B, ×63,500); lipopigment storage in the cytoplasm of a blood cell (C, ×40,000).

presence of characteristic cytosomes, in both non-neural and neural elements in several extracerebral tissues, has been reported over several decades [49,100], but is not a universal finding and so brain biopsy might still be considered where extracerebral tissue yields negative results [100]. Nonspecific lysosomal storage, such as lipopigment material, condensed osmiophilic bodies, etc., indicative of either lysosomal storage or cellular products of the ageing process, can be readily detected in different cell elements in the skin, skeletal muscle and rectal mucosa. It is far more difficult to detect specific NCL cytosomes such as RLP or FPP in the skin or skeletal muscle which may lead to false negative results, but NCL cytosomes can be identified with less difficulty in the cytoplasm of myenteric neurons in rectal biopsies. Therefore, we conclude that ultrastructural investigation of rectal tissue is the best diagnostic approach in ANCL, either to confirm the diagnosis according to clinical and pathological criteria, or to guide the molecular analysis of recently identified ANCL genes.

4.4.1. Adult autosomal-recessive type of NCL

Autosomal recessive ANCL encompasses a group of disease whose phenotypes span from classical Kufs A disease (characterised by progressive myoclonus epilepsy (PME), with ataxia and cognitive deterioration [101]) to other forms with earlier onset, prevalence of psychiatric symptoms, or late involvement, if any, of the visual system. To date, genetic–pathological correlations have been established for autosomal recessive ANCL forms, with mutations in *CLN1*, *CLN5*, *CLN6*, *ATP13A2* and *GRN* genes [13,15,54].

4.4.1.1. CLN1 — adult presentation. The first cases of adult NCL with PPT1 deficiency and GROD were reported in two sisters, presenting at the ages of 31 and 38 years, with psychiatric symptoms only [54]. The same patients (at age 56 and 54 years), developed visual, verbal, and cognitive losses, both had cerebellar ataxia and neither could walk without support. The causative mutations in the CLN1 gene were R151X and the novel missense mutation G108R.

Ultrastructural features in this variant disorder include storage cytosomes containing GROD. Extracerebral tissues have similar characteristics and storage distribution consistent with that seen in CLN1. Some adult patients, however, may present with GROD but neither PPT1 nor CTSD deficiency [24].

4.4.1.2. CLN5 — adult presentation. Two Caucasian patients with cognitive regression and visual loss, of late adolescence onset, had compound heterozygosity for mutations in CLN5. Both CVP and FPP were detected in skin biopsy [13].

4.4.1.3. CLN6 — adult presentation. Adult onset CLN6 disease is less rare than the previous forms. Patients have been described featuring the Kufs A phenotype. Visual function is not affected in the adult form, and no characteristic pattern can be observed by EP study. Phenotypic heterogeneity can occur within the same family. Both homozygous and heterozygous recessive mutations have been associated with CLN6 patients [15].

In adult onset CLN6 disease, lipopigment material and condensed osmiophilic bodies are frequently seen in different cell types in the skin as well as in the skeletal muscle (Fig. 24). FPPs are rare but their presence is a strong incentive to search for mutations in the *CLN6* gene. Ultrastructural analysis of rectal mucosa is more helpful in the diagnostic work-up. Along with autofluorescence in frozen or paraffin-embedded samples, ultrastructural investigations of myenteric neurons will reveal aggregates of FPP surrounded by a single membrane of lysosomal origin (Fig. 25). This specific inclusion is often present in lipopigment material. Other less specific storage both within the neuronal cytoplasm and in that of other cell types, observed in rectal biopsy material, has been described in post-mortem brain [15]. Vacuolated cells and dilated ER cisternae can be detected in both skin and rectal biopsies. EM studies of blood lymphocytes are not helpful.

4.4.1.4. CLN11. Homozygous mutations in the progranulin gene *GRN* have recently been reported in a family affected with adult onset progressive dementia and retinopathy. FPPs were detected in glandular and endothelial cells of the proband's skin [102]. To date, this family is the first and only one revealing adult NCL with retinopathy.

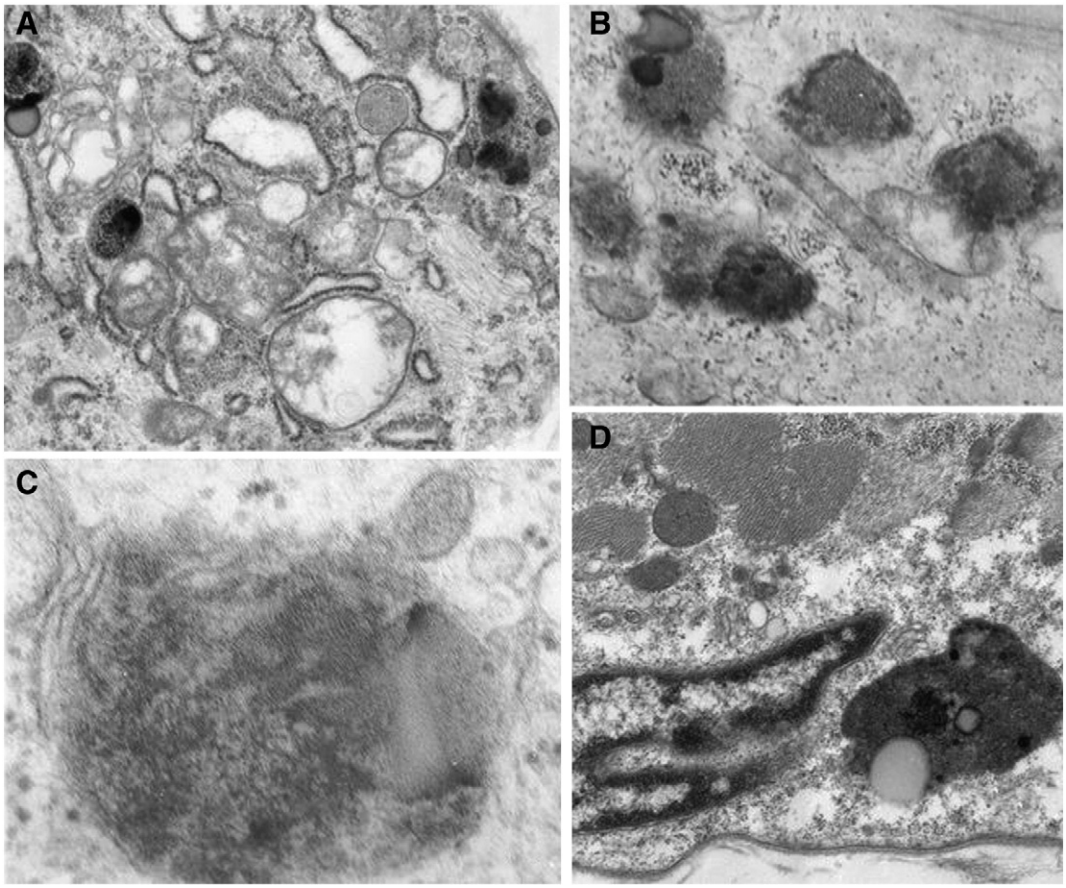

Fig. 24. *Adult CLN6*: Skin (A–C) and skeletal muscle (D) biopsies in two adult patients mutated in *CLN6*. Fibrocytic processes containing round osmiophilic deposits of different size (A and B) and stacks of osmiophilic material (B) embedded in a fine matrix and lined by a single membrane; note dilated ER cisternae in A (A, ×36,000; B, ×64,000); mixed population (granular elements and RLP) in a cytosome (C, ×135,000); lipopigment storage (granular material and lipid droplet) in the subsarcolemmal space of a skeletal muscle fibre (D, ×28,500).

4.4.1.5. Kufs B. The pathological features in extracerebral tissues of Kufs B cases secondary to mutations in the cathepsin F gene [103] are yet to be reported.

4.4.2. Adult autosomal-dominant type of NCL

Autosomal dominant adult onset NCL (Parry disease, CLN4) is a rare condition (only a few families have been described so far), characterised by generalised seizures, movement disorders and cognitive deterioration followed by progressive dementia. Clinically, the lack of myoclonus epilepsy and ataxia distinguish this disease from Kufs A disease, the more common form of ANCL. The disease is associated with heterozygous mutations of the *DNAJC5* gene which encodes a cysteine-string protein alpha acting in synaptic vesicles [29]. GRODs are the cytosomes observed in brain tissue and, to a lesser extent, in extracerebral biopsies (including blood lymphocytes; Fig. 26) [28,29].

4.5. Lipopigments in animals

To view ultrastructural findings of lipopigments in genetically different NCL species in the context of the overall ultrastructural spectrum of lipopigments, electron microscopic findings of lipopigments in spontaneous animal models have been tabulated (Table 2), in particular as such animal models may – in the future – be employed in therapeutic trials and, then, success or failure of treatment may not only be judged by results of clinical investigations but may also, especially in smaller animals, be further corroborated by morphological studies concerning increase or decrease in lipopigment load or alterations in lipopigment ultrastructure.

5. Outlook

Our knowledge of individual gene profiles and biochemistry of the NCL disorders is expanding and providing further clarification of the disease process. Examination of the morphology of storage products in the CNS and other tissue sites is already informative and of diagnostic

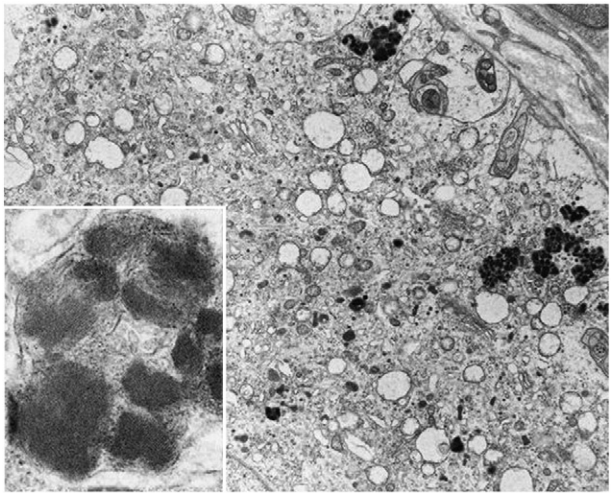

Fig. 25. *Adult CLN6 — rectum*: Osmiophilic storage material, either clustered or scattered in the cytoplasm of a myenteric neuron (×9000), made up of parallel stacks of membranes forming FPP (Insert, ×90,000).

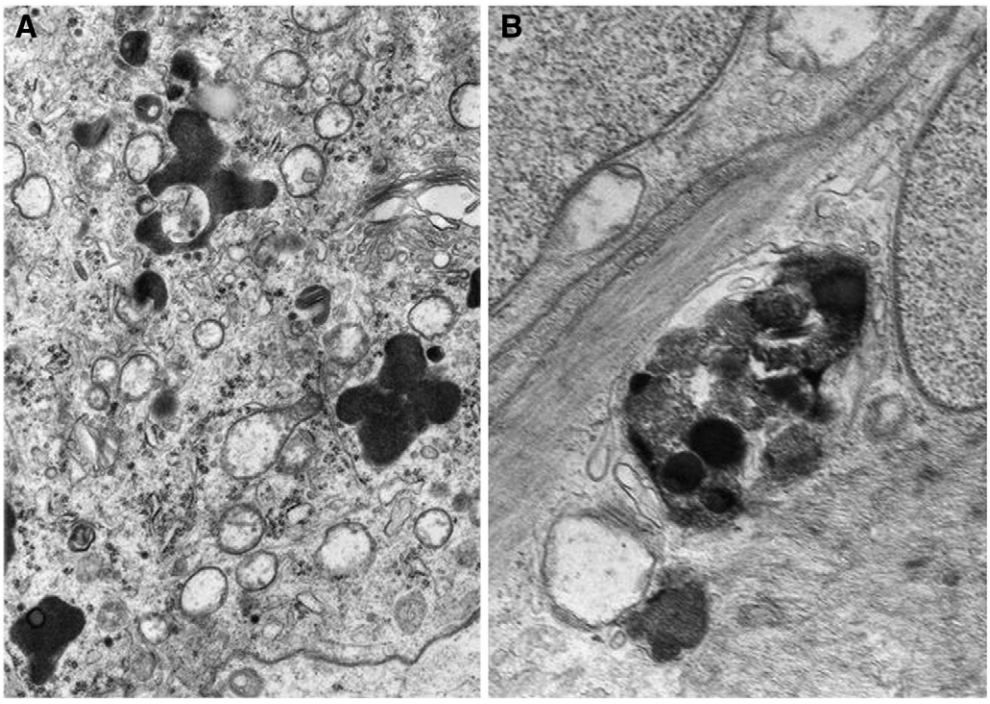

Fig. 26. *DNAJC5/CLN4*: Rectal (A) and skin (B) biopsies in an adult patient mutated in *DNAJC5*. GRODs scattered in the cytoplasm of a myenteric neuron (A, ×26,000); GROD-like elements associated with granular matrix in a smooth muscle cell (B, ×32,000).

value, but further investigation is required especially as newer gene disorders are being identified. The precise relationship between storage cytosomes and the profound CNS damage is unclear. Also, we do not have a clear reasoning as to why storage outside the nervous system does not appear to cause tissue destruction or corresponding physiological impairment.

Experimental work on animal models is providing some clues to these dilemmas since knowledge gained might be extrapolated to the human disease. However, without a detailed understanding of the mechanisms underlying each form of NCL disorder, successful treatment for this fatal disease will be hampered. There may be considerable value in retrospective analysis, combining modern morphological, immunocytochemical, molecular and EM methodology, to review and re-evaluate archived patient-derived samples.

As to future perspectives, it is unlikely that new brain biopsy material will become available. Prospective post mortem studies, however, since NCLs are invariably fatal diseases, may yet yield more information about selective or ubiquitous lipopigment formation as well as neuronal loss and early microglial activation. In view of the increasing availability of neuron-specific and intracellular antibodies, even against lysosomal proteins, a wealth of new data may be expected from future post mortem studies and, as brain tissue obtained by autopsy and biopsy is usually archived in paraffin blocks, these new techniques may be very gainfully employed retrospectively too.

Selective involvement of different grey matter regions has, so far, only rarely been demonstrated [9]. Increasing awareness of the neuronal periphery involved in NCL, marked by early microglial reactions and subtle neuropathology in mouse models, begs the question (on archival and future non-archival brain tissues) whether there is correspondence (or lack thereof) among different brain regions of different genetic human forms of NCL and different mouse models. EM studies of biopsied brain tissue, usually cerebral cortex, have focused on lipopigment formation and ultrastructure, largely for diagnostic purposes. A retrospective examination for research purposes may focus more thoroughly on structural damage; concentrating on synaptic contacts and the neuropathology of neuronal processes as well as features of glial pathology. Thus, prospects for neuropathological research investigations of human NCL brains are bright and promising and ought to be vigorously pursued.

Histopathological and EM examinations of extracerebral tissues and lymphocytes also remain essential adjuncts to the diagnostic workup. These techniques remain central to the differential diagnosis of NCLs from non-NCL neurodegenerative disorders and for determining which CLN mutation may be present or likely. Careful microscopical examination of lymphocytes for vacuolation, even where EM is not available, is a rapid, inexpensive, and non-invasive diagnostic tool.

Table 2
Ultrastructural profiles in spontaneous animal models of human NCL.
From Ref. [104].

Animal breed		EM/ultrastructure	Human gene equivalent
Sheep	New/South Hampshire sheep	cl, fp, ml, crystalloid	CLN6
	Swedish Landrace sheep	GROD	CLN10/CTSD
	Borderdale sheep	cl, ml with fp	CLN5
Cow	Devon cattle	cl, ml with fp	CLN5
Cat		Membranous whorls straight + curved profiles, ml	Unknown
Dog	American bulldog	gr	CLN10/CTSD
	American Staffordshire Terrier	Curved profiles	ARSG
	Australian Shepherd dog	"Modified" cl	CLN6
	Border Collie	fp, membranous, crystalloid	CLN5
	Dalmatian dog	fp, cl, lamellar	Unknown
	English Setter	fp, cl, lamellar	CLN8
	Longhaired Dachshund	cl	CLN2/TPP1
	Dachshund	gr	CLN1/PPT1
	Tibetan Terrier	Lamellar	ATP13A2
	Miniature Schnauzer	GROD	Unknown
Ferret		fp, cl, membranous	Unknown
Goat		fp, clm	Unknown
Horse		fp, cl, rl	Unknown
Mouse	ndf	fp, cl, membranous	CLN6
	mnd	rl, lamellar	CLN8
Pig		ml, cl, granular	Unknown

cl, curvilinear. clm, concentric-laminated-membranous. gr, granular. fp, fingerprint. GROD, granular osmiophilic deposits. ml, multilamellar. rl, rectilinear.

Whilst DNA probes remain prohibitively expensive narrowing the numbers of probes required is a major cost benefit. There remains also a large proportion of NCL cases for which gene mutations have yet to be established, which cannot be identified by molecular methodology.

Acknowledgement

We are grateful to Astrid Woeber for excellent editorial assistance.

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